THE DETERMINATION OF VITAMIN D WITH RADIOPHOSPHORUS [1]

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THREE FIGURES

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It is well recognized that various techniques for assaying vitamin D, whether biological, physical, chemical, or combinations of these, leave much to be desired. The biological techniques, usually dependent upon the use of the rat or chick as the test animals, present the difficulty that the variability in reaction of individual animals necessitates averaging the results of a considerable number of determinations. Chemical and physical methods are, by their nature, limited to concentrates which contain various reactants and consequently are lacking in specificity, and frequently in permanency of reaction as well. Furthermore, satisfactory vitamin D concentrates are not always obtainable as, for example, from starchy and cellulosic materials, from which vitamin D cannot be extracted quantitatively. Some of these difficulties have been presented in a recent review (Bills, '47).

Weissberger and Harris ('42) have expressed themselves unfavorably on the use of P^{32} for vitamin D assays partly because "numerous studies in which radiophosphorus was used failed to show a practical correlation." However, it

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appeared to us that the possible use of P^{32} was still an open question. In brief, our experimental efforts involved a quantitative comparison of the external radioactivity of rats which received a vitamin D preparation of unknown potency with that of other rats which received graded amounts of a standard reference preparation. In the technique as finally developed all measurements of radioactivity were made on the right forepaws of the living animals.

EXPERIMENTAL

Preparation of the rats and administration of test materials

Fifty- to 60-gm (21-day-old) Sprague-Dawley rats were allowed to develop rickets on ration 2965 (Steenbock and Black, '25). After 16 days rachitogenesis they were given orally, with a small syringe, a single dose of vitamin D_2 in 0.04 to 0.25 ml cottonseed oil. For the standard dose of 5 units the volume was usually 0.1 ml. For doses as low as 1.0 unit, or in excess of 10 units, solutions of respectively lower or higher concentrations were used.

Forty-eight hours after the administration of the vitamin, 0.25 ml of an isotonic saline solution (pH 5.0) containing approximately 20 μc of P^{32} were injected intraperitoneally. The P^{32} was essentially "carrier free," as it contained less than 10 μg P^{31} per dose. After the injection, the rats were continued on ration 2965 for 10 days without further treatment.

Determination of the external counts

At the end of the 10-day period "external counts" were taken on the anesthetized [3] rats held with their forepaws in a reproducible and immovable position between two brass platforms and beneath a mica-end-window Geiger counter. The lower platform carried a guide which held the right arm of the rat in such a position that the paw was spread out palm down and secured on a strip of Scotch tape stretched

[3] Nembutal.

over a raised area of the platform, between two spools. The G-M tube was mounted firmly over an opening in the upper platform in a holder equipped with shelves for the insertion of absorbers or standards for calibration. The distance between the mica window (35-mm diameter, 2.0 to 4.0 mg cm^2) and the lower platform was 5 cm.

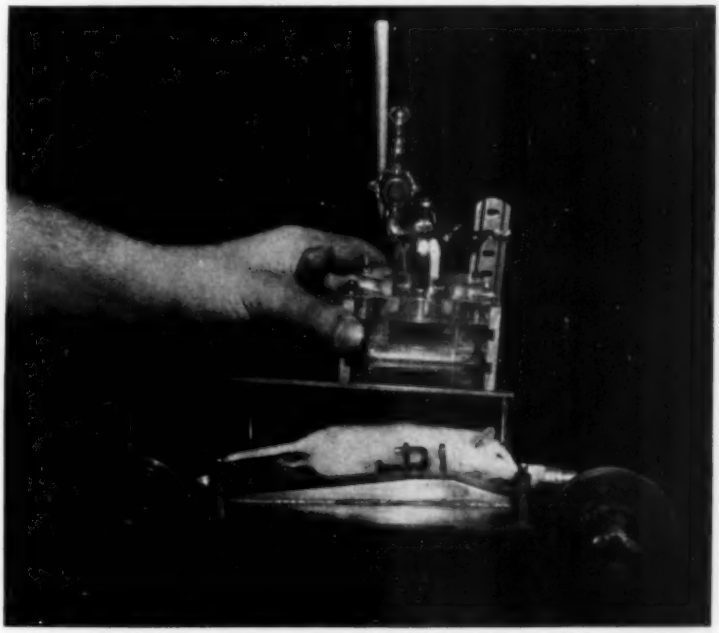

Fig. 1 This shows the lower platform of the rat holder on which the anesthetized rat has been placed with its right paw fixed on Scotch tape, and the upper platform carrying the Geiger counter which is about to be placed in position over the rat.

With the rat and counter in position (fig. 1), 100 second counts, which represented about 3,000 total impulses, were taken. After correction for background, dose, decay, and counter efficiency, they were recorded as counts per second $\times$ 100/64. As a precaution against building up interfering

"background radiation," the used area of Scotch tape was replaced with fresh tape from the spool after each determination. The correction for dose was based on the ratio of the actual dose to a dose of 20 μc, while the correction for counter efficiency was based on the ratio of a count obtained with a uranium standard to a similar count previously obtained and arbitrarily taken as a base. With these corrections and precautions, the results for separate experiments were comparable; the units used were arbitrary.

Response curves

With the technique described, two series of experiments on groups of 10 rats for each average value were carried out, using different doses of vitamin D. The results (fig. 2) reveal a linear semilogarithmic relation between the external count and the dose. In series I the increase over the control count (the lower limit of the curve) ranged from 38% for one unit to 145% for 20 units. In series II one-half unit gave an increase over the controls of 15% and 50 units, 160%. Apparently one-half unit approached the lower limit and 50 units the upper limit of sensitivity.

Previous to acceptance of the paws for the determination of radioactivity, determinations on other parts of the anatomy were made as well. The results obtained on the elbows and forearms were not satisfactory because of high variability and very small increases in count; on the other hand the forepaws, wrists and tails gave measurable reproducible results.

As was to be expected, most of the radioactivity was localized in bone. Not only did the soft tissues contribute relatively little to the total count, but they screened out much of the radiation. When, for example, the skin was stripped from the paws of rats which had received vitamin D, the counts were increased approximately 25%; when muscle and connective tissue were removed as well, the counts increased from 80 to 90%. A smaller increment was obtained with paws

from rats which had not received vitamin D because more of the injected radiophosphorus remained in their soft tissues.

The severity of rickets

In order to determine the period of rachitogenesis which would be most satisfactory, counts were taken on rats which

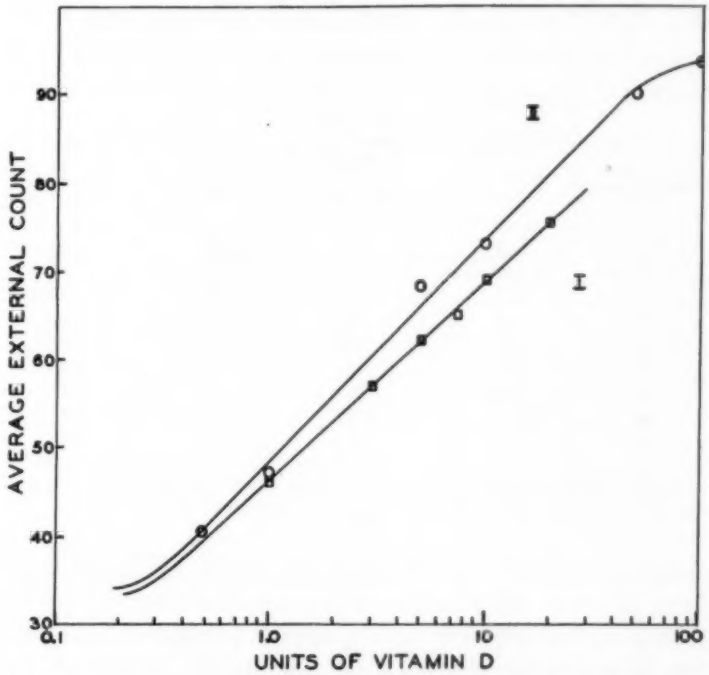

Fig. 2 Response curves: The effect of graded amounts of vitamin D on the external count of radioactivity in the forepaws of rats in two series of experiments. Each point represents the average of 10 rats.

had been kept on ration 2965 up to 27 days. The results (table 1) show a progressive decrease in the count for the controls as well as for the rats which had received vitamin D, but as the decrease was larger for the former than for the

vitamin D groups, a maximum differential was obtained after 16 days. All rats were therefore kept on ration 2965 for this period.

Time relations between the administration of vitamin D and P^{32}

In one series of experiments the degree of rickets, the time of administration of vitamin D, and the time of administration of P^{32} were varied collectively and individually. Some of these experiments were unavoidably complicated by the multiplicity of variables. However, the fact emerged that the time interval between the giving of vitamin D and the injection of radiophosphorus was not a critical factor in the response within the range studied (table 2). Forty-eight hours was accepted as adequate.

TABLE 1

The effect of the severity of rickets on the external count of rats with and without vitamin D

DAYS ON RATION 2965 BEFORE INJECTION OF P^{32}	EXPERIMENT NO.	AVERAGE EXTERNAL COUNT 10 DAYS AFTER INJECTION		% INCREASE DUE TO VITAMIN D
		Vitamin D group	Control group	
2	A-2	108 [1]	95	14
2	A-9	110	96	15
6	A-9	105	83	27
10	A-9	88	63	40
14	A-8	90	53	70
16	A-7	75	41	83
18	A-10	62	33	86
23	A-7	67	38	76
27	A-4	53	36	47

[1] One thousand units of D_3; all other groups, 5 units. In each case vitamin D was given 48 hours before the P^{32}. All groups contained 10 rats each.

Changes in the count with time

Radioactivity was detectable in the paws immediately after the injection of the P^{32}, and differences due to vitamin D

were detectable within 15 minutes after the injection. However, in practice, counts were not taken until the 10th day, since until then, or at least until the 8th day, there occurred not only a decrease in the variability of response by different animals but also the greatest differential in response (fig. 3).

Weight of the rats

The weight of the rats would be expected to have an inverse effect on the external counts but, probably because of

TABLE 2

The effect of varying the time interval between the administration of vitamin D and P^{32}

(Each group consisted of 10 rats.)

EXPERIMENT NO.	DAYS ON RATION BEFORE ADMINISTRATION OF 5 I.U. VITAMIN D	HOURS AFTER D BEFORE ADMINISTRATION OF P^{32}	EXTERNAL COUNT 10 DAYS AFTER THE INJECTION
A-6	17	144	62
A-6	19	96	67
A-6	21	48	71
A-6	21	96	62 [1]
A-1	21	6	58
A-1	21	24	59
A-1	21	48	61
A-5	21	48	59
A-5	22	24	56
A-5	23	6	51

[1] Counted at 8 days instead of 10.

the narrow range in weight of our animals, it was difficult to detect any such relation. As a precautionary measure we eliminated all rats which deviated more than 10 gm from the average weight of the group at the end of the period of rachitogenesis.

Single vs. divided doses of vitamin D

Whereas in the line test and in similar methods of assay, vitamin D often is given successively in small doses for 6

to 9 days, in the present method a single dose was found to give the best results. As was to be expected, the greatest effect was obtained when the highest concentration of the vitamin was present to react with the peripheral phosphorus when it had attained its highest specific activity. In a simple

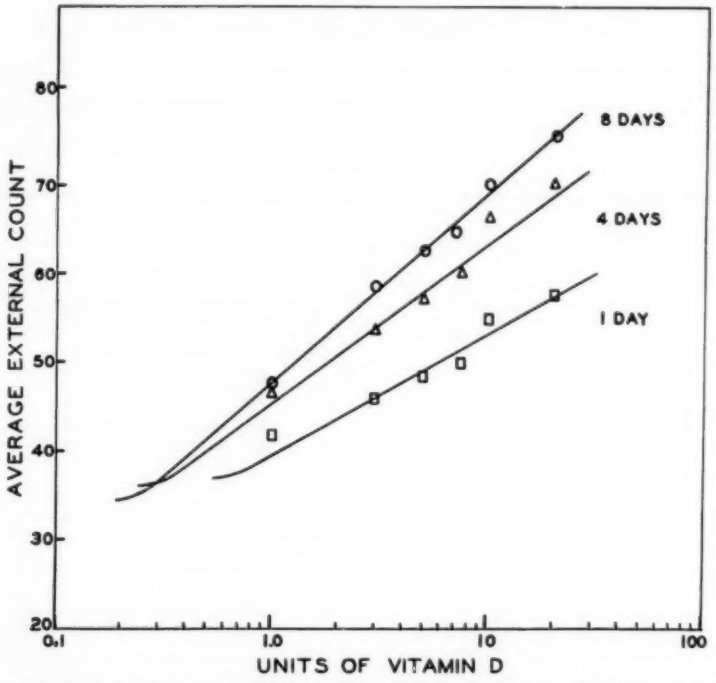

Fig. 3 The effect of varying the time interval between the injection of P^{32} and the taking of counts. In all cases vitamin D was given 48 hours before the injection of the P^{32}. Each point represents the average of 10 rats.

test, 5 units of vitamin D given in a single dose 48 hours before the injection of P^{32} gave a 30% increase 96 hours after the injection, while the administration of five-sixths of a unit daily for 6 days, with the P^{32} injected 6 hours after the first dose, gave an increase of only 14% 96 hours after the injection.

Accuracy of the method

The use of radiophosphorus as a measure of vitamin D is subject to the variabilities and errors ordinarily encountered in tracer work. With a counting period of 100 seconds, and with a total of approximately 3,000 counts, the probability of accuracy to within a fractional error of 0.04 was over 0.9. For comparison of counts taken at different times, corrections for counter variations were made by means of a uranium standard. This was satisfactory since the day-to-day variation ordinarily was not more than plus or minus 2 or 3%.

The size of the dose in microcuries was found to be reproducible to within 1 or 2%. Consecutive replacing and counting of the same rat showed that an individual count always could be duplicated to within 5% and usually to within 2 or 3%. Different operators consistently reproduced each other's results within 5%.

It is evident (fig. 2) that the slope of the response curve, while linear, was different for different series. Actually, over the course of a year, in 12 series including our preliminary tests, the increase in response of the experimental animals over that of the controls induced by 5 units of vitamin D ranged from 60 to 100%. In our more recent experiments this increase ranged from 80 to 100%. The possibility of obtaining a useful representative curve for continued use by any laboratory does not appear very promising. However, the alternative of establishing suitable standard references for any series of assays is not difficult, as it would be sufficient to establish reference points only at approximately the lower and upper limits of linearity; that is, with doses of one-half and 50 units of vitamin D, or within a somewhat narrower range if preferred.

The difference in response of individual animals in any given group in the two series reported was similar to that encountered in all of our work. In series I, figure 2, the average count for 10 animals which received one-half unit was

40; the individual values ranged from 34 to 47, with a standard deviation of 4.8. Similarly, with 10 units the average count was 73, with a range in values of 64 to 80 and a standard deviation of 5.4.

The data for series II, figure 2, were analyzed statistically by the methods of regression analysis according to Eisenhart ('39). Calculations were made of the limits within which 19 out of 20 additional observations would fall, i.e., at the 5% level of probability, with the following results: For a count of 50 resulting from an unknown dose, corresponding to 1.2 units of vitamin D on the curve, the true dose would lie between 0.80 and 1.75 units 95% of the time. For a count of 70 from an unknown dose, equal to 7.5 units on the curve, the confidence limits extend from 5.1 to 11.0 units. Similarly, for a count of 90, the limits extend from 30 to 74 units, compared to the value of 48 units read from the curve. Thus, at the 5% level the range of uncertainty extends approximately from -35% to $+50\%$. It so happens that this predicted degree of accuracy is almost identical with that found for the line test data of Coward ('38).

Limitations and advantages

It is evident that the method imposes a requirement for counting equipment and a replenishable supply of radioactive phosphorus. At the levels used, the handling and injection of the radioactive phosphorus pose no special problems. The total work involved is less than in the line test, since the removal of bones, staining, and the reading of the line are eliminated and the counts require only from two to three minutes of the operator's time for each rat. An aditional advantage of the method is that the animals need not be killed for the test, and therefore can still be used for other purposes.

Our findings on the extensive localization of radioactivity in bone, with a greater degree of variability in the soft tissues and accompanying interference in the external counts,

suggested that a comparison of radioactivity in bone would be preferable to determinations in the living animal. This, however, would involve the additional labor of dissecting out the bone, for example a femur, and a health hazard might be encountered through long exposure of the fingers if many assays were to be made.

A complication which is frequently encountered with the line test is that calcification is not always in evidence as a distinct line in the metaphyseal area, but as a diffuse accretion immediately contiguous to the diaphysis. This makes visual numerical evaluation very difficult, if not impossible. This difficulty is non-existent in the present method because in the measured area the exact location of the deposits is immaterial.

The fact that the new method is sensitive to as little as 0.5 I.U. of vitamin D, and to as many as 50 I.U., is also advantageous. The line test is limited to a range of approximately two to 8 units.

The fact that the vitamin D is given as a single dose might prove to be a disadvantage in the testing of low potency materials, because of their bulk. However, since the method is sensitive enough to detect small amounts, a smaller total dose than is required by other techniques would still be sufficient, or, if they are indispensable, a technique for multiple doses following each other in close sequence at the beginning of the test period could be worked out.

The effect of phosphorus in the test material was studied, since this is well known to yield high results by other assay techniques. O'Brien and Morgareidge ('39), using the line test, found that feeding 4 mg of phosphorus with each daily dose of vitamin D increased the apparent activity of the vitamin by a factor of 3.4. For the test made here, two groups of 10 rats each were given 5 units of vitamin D and a dose of P^{32} according to the usual procedure, but in addition one group was given orally 8 mg of phosphorus immediately after vitamin D had been given. There resulted no significant difference in the external counts of these

groups. If larger amounts of phosphorus are involved, suitable curves of reference obtained with supplements of various amounts of P^{31} could be provided.

SUMMARY

A technique for the bioassay of vitamin D with the use of parenteral radiophosphorus has been described. In this technique young rats are fed a rachitogenic ration (ration 2965) for 16 days. They are then given orally an unknown vitamin D preparation, in a dose ranging in expected potency from one-half to 50 units. Forty-eight hours later this is followed with an intraperitoneal injection of a trace dose of P^{32}. After 10 days of continued feeding of the rachitogenic ration the rats are anesthetized and the radioactivity of a forepaw of the living animal is determined. This is accomplished by placing the rat with a forepaw fixed in a reproducible position on Scotch tape under an end-mica-window Geiger counter. The average count for 10 rats which received the unknown vitamin D is compared with values of a standard reference curve obtained with doses of known potency. This technique gives results which compare in accuracy with well-known methods and offers some advantages.

ACKNOWLEDGMENTS

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STUDIES WITH SHEEP ON THE USE OF CHROMIC OXIDE AS AN INDEX OF DIGESTIBILITY OF RUMINANT RATIONS[1]

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ONE FIGURE

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INTRODUCTION

Recent studies in this laboratory have shown that chromic oxide (Cr_2O_3) may be successfully used as an index of digestibility of the dry matter in the diets of three simple-stomached species. A close agreement between digestion coefficients obtained by the conventional method and by the Cr_2O_3 method was noted by Schürch, Lloyd and Crampton ('50) in the case of rats, by Irwin and Crampton ('51) in the case of humans, and by Lloyd ('51) for swine. Successful use of the method was also reported by Barnicoat ('45) with swine, and by Kreula ('47, '50) and Virtanen ('50) with humans.

On the other hand, the results recorded in the literature on the use of Cr_2O_3 as an index of dry matter digestibility with ruminants are not conclusive. Hamilton et al. ('27–'28), working with sheep and steers, reported an agreement between dry matter digestion coefficients calculated by the Cr_2O_3 method and by the conventional "time collection" method, but that this could be obtained only by prolonging the collection period. In New Zealand Barnicoat ('45) found that the results with the Cr_2O_3 method with both sheep and calves were

[1] Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Quebec, Canada. Journal Series No. 290.

usually significantly lower than those obtained by the conventional method. However, satisfactory results with Cr_2O_3 have been reported by Skulmowski et al. ('43) with sheep and horses, by Olsson, Kihlen and Cagell ('49) with horses, and by Kane et al. ('50) with cows.

In an attempt to devise some practicable method of employing Cr_2O_3 as an index in digestion trials, we have carried out a series of studies with sheep, the results of which are herein reported.

EXPERIMENTAL

Animals and rations

Four Cheviot ewes were penned individually in digestibility crates. In a series of three trials, half the sheep eventually received a ration of chopped Timothy hay and the others a mixture of 65% chopped Timothy hay plus 35% medium ground oats. Cr_2O_3 was included in both cases as 1% of the ration.

Method of Cr_2O_3 administration

Administration of the Cr_2O_3 in gelatin capsule form proved inconvenient, uncertain and laborious. This was avoided in the case of the hay-grain mixture by incorporating the index into the ground oats portion of the ration. The ground oats-Cr_2O_3 combination was mixed, dampened with water and dried in an air oven at 90°C. This procedure caused the Cr_2O_3 to adhere closely to the ground oat particles, and prevented sifting out of the index material.

In the case of the all-roughage ration, it was felt that the mixing of relatively large amounts of the index with a small amount of a ground feed to form a tidbit, relished and readily eaten by the sheep, might solve the Cr_2O_3 administration problem. Thus Cr_2O_3, medium ground oats and crude molasses, in the proportions by weight of 100 : 218 : 32 were mixed, moistened with water, pelleted into half-inch lengths, and dried. The quantity of concentrate required to supply 1%

Cr_2O_3 in the ration (about 25 gm per day) was so small that its content of ground oats and molasses did not measurably affect the digestibility of the roughage.

Feeding practice

Each sheep was brought to a constant level of complete daily consumption of its ration. The Cr_2O_3, fed once daily in trials I and II, was accompanied by the daily allowance of hay or of hay plus grain. When fed twice daily in trial III, it was accompanied at each feeding by half the daily ration allowance. Water and iodized salt were available at all times.

Six-day preliminary feeding periods were used, followed by 13-day periods during which Cr_2O_3 was included in the ration.

Feces collections

Quantitative feces collections were made in all trials for the 13 days following the 6-day preliminary feeding of the diets, and the data used to calculate dry matter digestibility by the conventional "time collection" method.

Within this 13-day period, and beginning on the 7th day (i.e., following 6 days of Cr_2O_3 feeding), sub-sampling of feces was done in all trials in such a manner that separate one-fifth aliquots each of morning, noon and evening voidings were taken and separately composited. In addition, in trials II and III "random" samples were "picked up" over a 4-day period for each sheep. These represented a close approach to the practical use of the index method.

In trial III a quantitative feces collection was carried out for the 13 days of Cr_2O_3 feeding and continued for a further 7 days to permit a complete record of Cr_2O_3 excretion rate.

RESULTS

Digestion coefficients

Dry matter digestion coefficients, determined both by the conventional "time collection" method and by the Cr_2O_3 method, are shown in table 1.

TABLE 1

Per cent digestibility of dry matter of timothy hay and of timothy hay plus ground oats by 4 sheep

SAMPLING METHOD	TRIAL I: Hay + grain + Cr_2O_3 (fed once daily)				TRIAL II: Hay + Cr_2O_3 pellets (fed once daily)				TRIAL III: Hay + Cr_2O_3 pellets (fed twice daily)		Hay + grain + Cr_2O_3 (fed twice daily)	
	Sheep				Sheep				Sheep			
	no. 1	no. 2	no. 3	no. 4	no. 1	no. 2	no. 3	no. 4	no. 1	no. 2	no. 3	no. 4
Conventional method	59	60	57	58	43	42	45	44	47	45	57	55
Cr_2O_3 method												
Composited 7-day morning collections	56	58	59	61	22	26	27	26	39	38	57	54
Composited 7-day noon collections	59	58	59	58	34	31	31	26	38	41	58	57
Composited 7-day evening collections	55	55	49	59	33	19	32	21	40	46	60	58
Random sample — 9th day					34	29	30	41	43	41	56	54
Random sample — 10th day					36	25	42	45	40	41	56	54
Random sample — 11th day					38	27	43	45	35	36	57	53
Random sample — 12th day					36	38	30	32	42	43	60	56
Average of 4-day period					36	30	36	41	40	40	57	54

It may be seen from these data that when roughage comprised the whole ration for sheep, the Cr_2O_3 method gave variable and low coefficients as compared to those determined by the conventional method. The twice-daily administration of the index material (sheep 1 and 2, trial III) resulted in slightly better agreement between the determined digestion coefficients but still was far from satisfactory.

On the other hand, when a ground grain comprised 35% of the sheep ration, and the Cr_2O_3 was incorporated into this part of the diet, digestion coefficients were in agreement with the conventionally-determined values.

TABLE 2

Recovery of Cr_2O_3

SHEEP	RATION	RECOVERY
		%
1	Roughage + Cr_2O_3 pellets	85
2	Roughage + Cr_2O_3 pellets	87
3	Roughage + grain + Cr_2O_3	98
4	Roughage + grain + Cr_2O_3	99

The coefficients calculated separately for the morning, noon and evening collections showed some variability. This is thought to be of no particular consequence, however, since the practical application of this method would call for the compositing of appropriate daily feces aliquots over a 4- or 5-day period, of which the data for the random sampling are illustrative. The values for this procedure are practically identical with those determined by the conventional method for the two sheep receiving the hay-grain ration (sheep 3 and 4, trial III).

Cr_2O_3 recovery

Recovery of Cr_2O_3 during the 13 days of Cr_2O_3 administration plus the 7 subsequent days gave the results shown in table 2.

It thus appears that on a roughage diet the Cr_2O_3 fed in a highly concentrated pellet is partially retained somewhere in

the digestive tract. It may eventually be completely excreted. This retention would account for the low dry matter digestion coefficients obtained when the Cr_2O_3 concentrate was used.

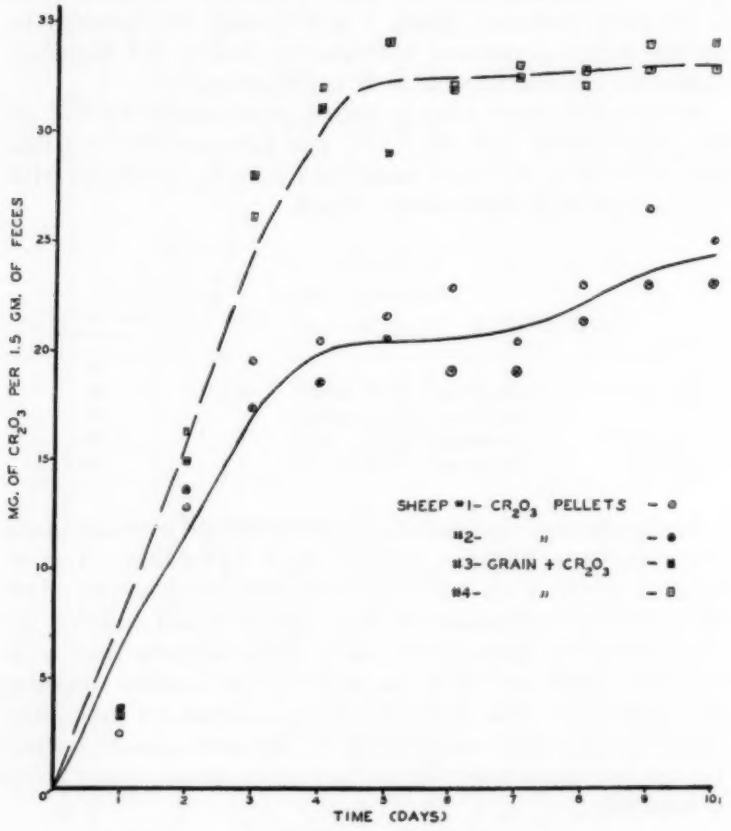

Fig. 1 Cr_2O_3 excretion over 10-day period.

On the other hand, when the index was mixed with a portion of the ration (i.e., in the grain), it was almost completely recovered within 7 days after its administration was discontinued.

Rate of Cr_2O_3 excretion

The excretion curves for Cr_2O_3 are shown in figure 1. It appears that when Cr_2O_3 is mixed with some portion of the ration, a minimum period of 5 days is required after the initial administration before the concentration of the index reaches a constant or maximum level in the feces. When fed in a concentrated form, however, the rate of excretion of Cr_2O_3 is slower and a minimum period of 9 days seems to be necessary to bring the fecal index concentration to a maximum, and there is no constant excretion level established.

DISCUSSION

The results of these trials indicate that Cr_2O_3 may be successfully used in determining the digestibility of a sheep ration provided the index material can be premixed with some ground component of the ration, but not when it is given separately, as is necessary when the ration is composed of dry roughage only.

These findings make it possible to reconcile the conflicting conclusions of two groups of workers. Barnicoat ('45) obtained consistently low digestion coefficients with sheep and calves when the Cr_2O_3 was given in a gelatin capsule, a means of administration comparable to the pelleted Cr_2O_3 concentrate employed in our work. However, the successful use of the method with cows, as reported by Kane et al. ('50), involved the incorporation of the Cr_2O_3 into the ground grain portion of an alfalfa-corn silage-grain ration.

The low average recovery of Cr_2O_3 (86%) for the sheep receiving the index in a concentrated form suggests that it was retained in some part of the digestive system. Barnicoat ('45) reported such a retention in the stomach folds of a lamb. However, the high average recovery of Cr_2O_3 (98.5%) for the other sheep indicates that when mixed with the grain the Cr_2O_3 remained with the ingesta, and was thus carried through the digestive tract.

Barnicoat reported that only two to 4 days elapsed before the excretion of Cr_2O_3 became approximately constant. Our results suggest that a minimum period of 5 days is required before any fecal samples should be retained for digestibility studies with adult sheep.

CONCLUSIONS

These studies indicate that the Cr_2O_3 method of determining digestibility of rations by sheep may be successfully employed provided the ration includes ground feed with which the Cr_2O_3 may be premixed.

Not less than 5 days following first ingestion of the index material must elapse before feces collection for analysis should be saved.

Random samples taken daily for 4 days and composited for each animal are satisfactory for determining digestibility.

On an unground all-roughage ration, Cr_2O_3 administered as such tends to be partially retained in the digestive tract, thus leading to unreliable and low estimates of digestibility.

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THE RELATION OF VITAMIN B_{12} AND FOLACIN TO THE UTILIZATION OF CHOLINE AND ITS PRECURSORS FOR LIPOTROPISM AND RENAL PROTECTION IN RATS

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THREE FIGURES

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In a previous report (Schaefer, Salmon, Strength and Copeland, '50), it was shown that the incidence and severity of renal damage in weanling rats receiving a diet low in choline and methionine were markedly reduced when the diet was supplemented with either folacin or vitamin B_{12}, and that when both were added the effect was additive. Continued studies have been made on the possible role of vitamin B_{12} and folacin in the utilization of choline or choline precursors for the maintenance of normal liver fat, prevention of renal damage, and promotion of growth. It was noted that for lipotropism both vitamin B_{12} and folacin were required, in contrast to the partial protection against renal damage provided by either vitamin B_{12} or folacin (Schaefer, Salmon and Strength, '50).

The purpose of this paper is to report the following: (1) that for lipotropism and protection against renal damage in rats, vitamin B_{12} and folacin increase the efficiency of choline, betaine, DL-methionine, dimethylaminoethanol, and methylaminoethanol; (2) that the triethylhomologue of choline appears to be an antimetabolite of choline; and (3) that vitamin B_{12} and folacin appear to be essential for the biological synthesis of choline.

EXPERIMENTAL

Weanling rats of the Alabama Experiment Station (AES) strain weighing 40 to 50 gm were placed in individual cages and uniformly grouped with respect to number, weight, sex and litter. In general, each treatment effect was determined on 4 rats per group and repeated one to 4 times. Feed and water were supplied ad libitum unless otherwise indicated. The basal diet contained extracted peanut meal 30 (50% protein) [1], sucrose 39.5, extracted casein 6 [2], salt mixture (undried) 4.4 [3], L-cystine 0.1, cod liver oil 1, and lard 19%. Vitamins were added (milligrams per kilogram of diet) as follows: thiamine HCl 2, pyridoxine HCl 2, riboflavin 4, calcium pantothenate 10, niacin 20, *i*-inositol 200, alpha-tocopherol 25, alpha-tocopherol acetate 25, 2-methyl-1,4-naphthoquinone 5. The total methionine and choline contents of the diet were 0.30% and 0.005%, respectively. In the later experiments a more purified diet was used which contained peanut meal and casein that had been further purified by 7 additional one-hour, hot 95% methanol extractions. By this procedure the choline concentration of the peanut meal was reduced from 0.17 mg per gram to 0.005 mg or less per gram.

All rats were necropsied after death or at the end of a 14-day experimental period; kidneys were examined for pathological lesions and liver fat determinations were made. Liver fat was expressed as per cent total ether-extractable material on a dry weight basis. For the determination of lipotropic activity wherever comparisons were made between treatments, it was required that maximum body weight gains be made by all groups. If not, the experiment was repeated using controlled paired feeding.

The responses to varying levels of dietary choline or choline substitutes were measured by use of three criteria; namely, body weight gain, maintenance of normal liver fat, and prevention of renal damage. In the discussion of these

[1] Engel, R. W., J. Nutrition, 1948, *36:* 739.
[2] Salmon, W. D., J. Nutrition, 1947, *33:* 155.
[3] See footnote 2.

experiments the term "choline equivalent" is used; this is based upon the hypothesis that the compounds were utilized for the biological synthesis of choline. One mole of aminoethanol, or methylaminoethanol or dimethylaminoethanol, when supplied with three, two, and one methyl groups, respectively, were considered equal to one mole of choline. For the purpose of choline synthesis, three moles of betaine HCl or DL-methionine were considered equivalent to one mole of choline. When dimethylaminoethanol was used alone, three moles were considered equivalent to two moles of choline.

RESULTS

The interrelationship of vitamin B_{12}, folacin, and choline in lipotropism

The ineffectiveness of vitamin B_{12} or folacin as lipotropic substances when fed singly to rats receiving the basal diet supplemented with 0.10% choline chloride is shown by the results in table 1. Supplementing the diet with both folacin and vitamin B_{12} reduced liver fat from an average of 43% to 18%.

Since both vitamin B_{12} and folacin were essential for the reduction of the dietary choline requirement for lipotropism, in subsequent experiments both vitamin B_{12} and folacin were either omitted or included in the diet. Supplementation of the diet with vitamin B_{12} and folacin markedly suppressed the accumulation of fat in the livers of rats fed subprotective levels of choline (table 1). In general, the liver fat of the rats fed the basal diet supplemented with 0.08% and 0.12% choline chloride and also with vitamin B_{12} concentrate and folacin was equal to that of rats receiving 0.12% and 0.16% choline chloride, respectively, without supplements of vitamin B_{12} and folacin. Since these 4 treatments supported maximum growth, protected against renal damage, and gave a marked reduction in liver fat, they were used as a basis for comparing choline to choline precursors for lipotropic activity.

Choline chloride fed at the 0.20% level was approximately as effective for the maintenance of normal liver fat as was 0.20% choline chloride plus vitamin B_{12} and folacin. The lipotropic activity of vitamin B_{12} and folacin appears to consist of a choline-sparing effect, which is manifest at subprotective levels of choline.

TABLE 1

Interrelationship of vitamin B_{12}, folacin and choline in lipotropism

DIETARY SUPPLEMENT			NO. OF RATS	AVERAGE BODY WT. GAIN	LIVER ANALYSIS		INCIDENCE OF KIDNEY DAMAGE
Choline	Vitamin B_{12}	Folacin			Fat (dry basis)	Water	
%	*µg/kg*	*mg/kg*		*gm/2 wks.*	%	%	%
0.04	0	0	28	19	. .	. .	100
0.06	0	0	16	44			69
0.08	0	0	8	49	60	54	13
0.10	0	0	8	55	43	63	0
0.12	0	0	8	55	31	62	0
0.16	0	0	4	61	17	70	0
0.20	0	0	8	63	14	70	0
0.04	2	30	12	63	58	53	0
0.06	2	30	8	55	48	57	0
0.08	2	30	8	53	28	64	0
0.10	2	30	8	58	18	64	0
0.12	2	30	8	53	17	67	0
0.16	2	30	4	62	14	68	0
0.20	2	30	8	56	12	69	0
0.10	10	0	8	50	49	57	0
0.10	0	150	8	53	39	60	0

The activity of betaine HCl and DL-*methionine in lipotropism and in protection against renal damage*

Vitamin B_{12} and folacin added to the basal diet supplemented with varying levels of betaine or DL-methionine exerted a marked sparing effect on the requirement of these compounds for the promotion of maximum growth, prevention of renal damage and lipotropism (table 2). Supplementation

of the diet with betaine HCl or DL-methionine at the 0.08% choline chloride equivalent level, without vitamin B_{12} and folacin, resulted in an incidence of renal damage of 100% (table 2) as compared to 13% for choline chloride (table 1). When the diet was supplemented with vitamin B_{12} and folacin, three moles of betaine HCl or DL-methionine were as effective as one mole of choline for protection against renal damage and lipotropism.

TABLE 2

The activity of betaine and methionine

DIETARY SUPPLEMENT			NO. OF RATS	AVERAGE BODY WT. GAIN	LIVER ANALYSIS		INCIDENCE OF KIDNEY DAMAGE
Methyl donor	Choline cl. equiv.	Folacin and vitamin B_{12}[1]			Fat (dry basis)	Water	
	%			*gm/2 wks.*	%	%	%
Betaine HCl	0.08	0	8	20	..		100
Betaine HCl	0.12	0	8	48	30	63	0
Betaine HCl	0.16	0	8	53	16	69	0
Betaine HCl	0.06	+	6	60	51	60	0
Betaine HCl	0.08	+	8	54	24	67	0
Betaine HCl	0.10	+	8	50	18	69	0
DL-Methionine	0.08	0	8	19	..	..	100
DL-Methionine	0.12	0	8	43	36	62	0
DL-Methionine	0.16	0	8	48	25	66	0
DL-Methionine	0.08	+	8	49	33	63	0
DL-Methionine	0.10	+	8	52	21	66	0

[1] Vitamin B_{12} fed at 30 μg and folacin at 2 mg per kilogram diet.

The activity of dimethylaminoethanol and methylaminoethanol in lipotropism and promotion of growth

Neither dimethylaminoethanol nor methylaminoethanol fed to rats at varying levels was equal to choline for the promotion of growth and maintenance of normal liver fat (tables 3 and 1). In the absence of vitamin B_{12} and folacin, increasing the level of methylaminoethanol or dimethylaminoethanol resulted in a depression of body weight gain. When these compounds were fed at a choline chloride equivalent level of

0.16% (without vitamin B_{12} and folacin), average liver fat values were 36 and 26%, respectively (table 3), as compared to 17% when choline was fed (table 1).

TABLE 3

The activity of dimethylaminoethanol and methylaminoethanol

DIETARY SUPPLEMENT			NO. OF RATS	AVERAGE BODY WT. GAIN	LIVER ANALYSIS		INCIDENCE OF KIDNEY DAMAGE
Methyl donor	Choline cl. equiv.	Folacin and vitamin B_{12} [1]			Fat (dry basis)	Water	
	%			*gm/2 wks.*	%	%	%
Dimethyl-aminoethanol	0.12	0	6	46	28	68	0
Dimethyl-aminoethanol	0.16	0	6	42	26	69	0
Dimethyl-aminoethanol	0.40	0	8	32	24	69	0
Dimethyl-aminoethanol	0.08	+	4	45	46	60	0
Dimethyl-aminoethanol	0.10	+	8	52	32	66	0
Dimethyl-aminoethanol	0.20	+	6	65	18	68	0
Dimethyl-aminoethanol	0.40	+	4	46	17	69	0
Methyl-aminoethanol	0.08	0	8	31	38	67	13
Methyl-aminoethanol	0.12	0	8	20	36	68	13
Methyl-aminoethanol	0.16	0	8	10	36	70	25
Methyl-aminoethanol	0.20	0	8	13	29	64	25
Methyl-aminoethanol	0.06	+	4	51	41	64	0
Methyl-aminoethanol	0.08	+	4	39	38	67	0
Methyl-aminoethanol	0.12	+	6	48	24	67	0
Methyl-aminoethanol	0.20	+	8	30	20	67	0

[1] Thirty micrograms crystalline vitamin B_{12} and 2 mg folacin per kilogram of diet.

The addition of vitamin B_{12} and folacin reduced liver fat. The activity of dimethylaminoethanol was considerably greater than that of methylaminoethanol. When fed at the 0.08 or 0.10% choline chloride equivalent level, average liver fat values were 46 and 32%, respectively, for dimethylaminoethanol (table 3), as compared to 28 and 18%, respectively, for choline chloride.

TABLE 4

The toxicity of triethylcholine

DIETARY SUPPLEMENT			NO. OF RATS	AVERAGE BODY WT. GAIN	LIVER ANALYSIS		INCIDENCE OF KIDNEY DAMAGE
Triethyl choline (choline cl. equiv.)	Choline cl.	Folacin and vitamin B_{12}[1]			Fat (dry basis)	Water	
%	%			*gm/2 wks.*	%	%	%
0.06	0	+	4	20	42	50	50
0.08	0	+	4	32	48	58	0
0.20	0	+	4	16	33	59	25
0.20	0	0	8	2	40	58	63
0.04	0.04	+	4	60	81	52	0
0.06	0.04	+	8	54	38	61	0
0.08	0.04	+	4	44	34	63	0
0.10	0.04	+	4	38	39	59	25

[1] Thirty micrograms crystalline vitamin B_{12} and 2 mg folacin per kilogram of diet.

The toxicity of triethylcholine

The triethylhomologue of choline (triethyl-β-hydroxyethylammonium hydroxide) fed to weanling rats seemingly functioned as an antimetabolite of choline (table 4). When the basal diet was supplemented with 0.04% to 0.20% choline chloride, vitamin B_{12}, and folacin, maximum body weight gain (53 to 63 gm in two weeks) was attained (table 1); however, supplementing the diet with triethylcholine greatly arrested body weight gain and resulted in renal damage and fatty livers. The addition of choline chloride to replace part of the triethylcholine aided in promoting growth and in protection against renal damage. However, as the level of tri-

ethylcholine was increased, and the choline chloride supplement maintained at 0.04%, the body weight gain was again depressed and liver fat values remained high.

The effect of vitamin B_{12} and folacin on utilization of methylaminoethanol and methionine in lipotropism

Continued studies on the function of vitamin B_{12} and folacin in the utilization of choline precursors in lipotropism and

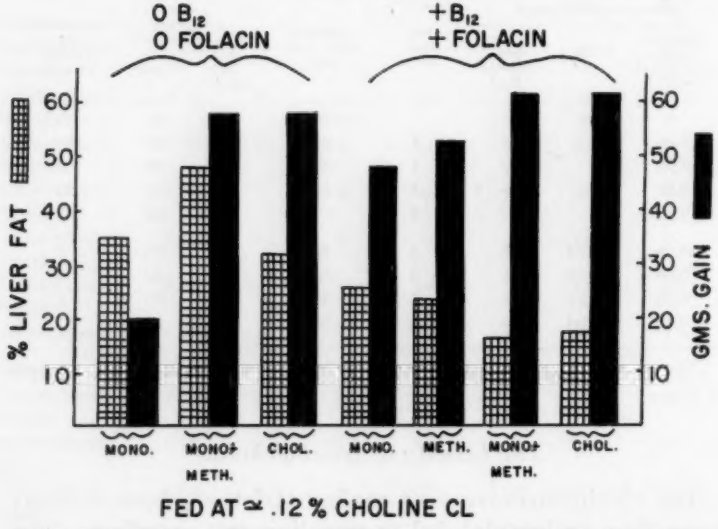

Fig. 1 The activity of methylaminoethanol and DL-methionine. Each treatment was repeated with 4 rats per group. One mole of methylaminoethanol and two moles of DL-methionine replaced one mole of choline. When methylaminoethanol was fed singly, three moles were used to replace one mole of choline. + B_{12} + folacin = 30μg of crystalline vitamin B_{12} and 2 mg folacin per kilogram of diet. Mono. = methylaminoethanol. Meth. = DL-methionine.

protection against renal damage indicated the need for a more purified diet in which the choline, choline precursors, vitamin B_{12}, and folacin content could be reduced to amounts lower than those contributed by the basal diet. In the follow-

ing experiments the more purified diet, in which the casein and peanut meal were thoroughly extracted with methanol, was used.

The original basal diet supplemented with betaine or DL-methionine at a choline equivalent level of 0.08%, plus vitamin B_{12} and folacin, completely protected against renal damage, whereas the more purified diet supplemented with the

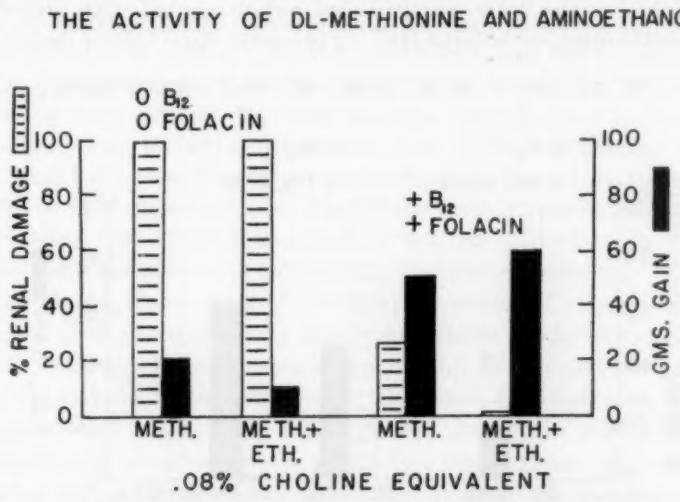

Fig. 2 Each treatment was repeated three times with 4 rats per group. Three moles DL-methionine were added in place of one mole of choline. Eth. = aminoethanol added at one mole to replace one mole of choline. Meth. = DL-methionine added at three moles to each mole of aminoethanol. + B_{12} + folacin = 30μg crystalline B_{12} and 2 mg folacin per kilogram of diet.

same level of betaine or DL-methionine resulted in 100% and 30% renal damage, respectively.

Typical data obtained by the use of this more purified diet are illustrated in figure 1. When vitamin B_{12} and folacin were added to the diets that were supplemented with methylaminoethanol (one mole) and DL-methionine (two moles) at a choline equivalence of 0.12%, the lipotropic activity of this combination was equal to that of choline. In the absence of vitamin

B_{12} and folacin, the lipotropic activity was not equal to that of choline. These results indicate that vitamin B_{12} and folacin are involved in the biological synthesis of choline from methylaminoethanol and methionine.

The role of vitamin B_{12} and folacin in the methylation of aminoethanol by the rat

Rats fed the more purified diet supplemented with either DL-methionine, or betaine HCl, or DL-methionine (three moles)

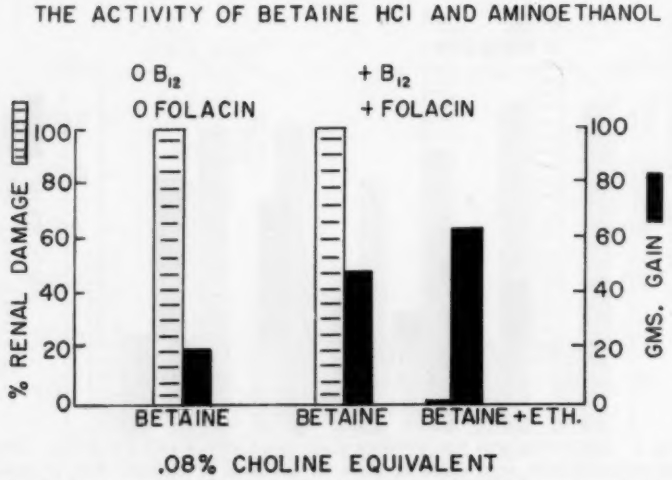

Fig. 3 Each treatment was repeated three times with 4 rats per group. Three moles of betaine HCl were added in place of one mole choline. Eth. = aminoethanol added at one mole to replace one mole choline. + B_{12} + folacin = 30 μg crystalline vitamin B_{12} and 2 mg folacin per kilogram of diet.

and aminoethanol (one mole) at a choline chloride equivalent level of 0.08%, developed 100% renal damage. Supplementation of the purified diet with vitamin B_{12} and folacin, and either DL-methionine or betaine HCl, resulted in 30 and 100% renal damage, respectively. The further addition of aminoethanol to these diets gave complete protection against renal damage and supported maximum growth. Typical data are

illustrated in figures 2 and 3. These results indicate that vitamin B_{12} or folacin, or both, are essential for the synthesis of choline from aminoethanol and methionine or betaine.

DISCUSSION

It appears that vitamin B_{12} and folacin are involved in the utilization of labile methyl, and choline and methionine precursors, for the synthesis of choline and methionine. Jukes et al. ('50) reported that vitamin B_{12} was essential for the methylation of homocystine for growth in chicks fed a methionine-deficient diet. Schaefer, Salmon and Strength ('50) reported that vitamin B_{12} was essential for the synthesis of choline from methylaminoethanol, and either methionine or betaine for prevention of perosis and production of maximum body weight gain in chicks. The present study employing rats indicates that vitamin B_{12} and folacin may function in the biological synthesis of choline from various precursors.

Since the observation of the sparing action of vitamin B_{12} on the choline requirement of rats and chicks (Schaefer et al., '49), reports by Stekol and Weiss ('50b), Welch and Sakami ('50), and du Vigneaud et al. ('50) have demonstrated the synthesis of biologically labile methyl groups *in vivo*. The data herein reported do not refute this observation; however, under the experimental conditions employed, the amount of biological synthesis of labile methyl was insufficient to meet the requirements of weanling rats for prevention of renal damage, maintenance of normal liver fat and promotion of growth.

Drill and McCormick ('49) reported that a vitamin B_{12} concentrate exerted a lipotropic effect in rats maintained on a high fat diet. In the present studies it was noted that neither vitamin B_{12} nor folacin, when added singly to the basal diet supplemented with a suboptimum amount of choline, was effective in preventing fatty livers; however, the addition of both vitamin B_{12} and folacin resulted in a marked lipotropic activity. The lack of lipotropic activity of either vitamin B_{12}

or folacin when the other is omitted from a diet deficient in choline indicates that both vitamins may have a function in the synthesis or utilization, or both, of choline. Supplementing the basal diet with vitamin B_{12}, folacin and 0.10% choline chloride maintained liver fat at a level approximately equal to that obtained when 0.16% choline chloride was fed without vitamin B_{12} and folacin. This reduction of the choline requirement by vitamin B_{12} and folacin may be due either to the biological synthesis of additional choline or to increased utilization of the choline.

Three moles of betaine HCl or DL-methionine were comparable to one mole of choline chloride for protection against renal damage and lipotropic activity when the basal diet was supplemented with vitamin B_{12} and folacin. This confirms the view of Welch ('50) that choline per se is required for these functions and that only one methyl group of betaine or the methyl of methionine may be of significance in choline synthesis.

In a comprehensive review of transmethylation reactions Jukes ('47) suggested that methylaminoethanol and dimethylaminoethanol serve as methyl acceptors and are incapable of donating methyl groups. The results of our experiments show that vitamin B_{12} and folacin greatly increased the activity of these compounds for lipotropism, prevention of renal damage and promotion of growth. Neither methylaminoethanol nor dimethylaminoethanol was equal to choline on a methyl basis; however, dimethylaminoethanol was more effective than methylaminoethanol. The increased activity of these choline precursors may be interpreted as being due to the synthesis of methyl and its utilization for choline synthesis. The results with methylaminoethanol indicate insufficient biological synthesis of methyl groups and the inability of this compound to donate methyl efficiently for the complete methylation of the aminoethanol moiety to choline.

Triethylcholine was reported to be a lipotropic agent by Channon and Smith ('36). Keston and Wortis ('46) reported

that triethylcholine is toxic to mice and that the toxicity can be alleviated by choline. Under the conditions of our experiments, triethylcholine cannot be classified as a lipotropic agent capable of replacing choline.

Stekol and Weiss ('50a) reported that triethylcholine inhibited the growth of rats and that this inhibition was alleviated by choline. Since McArthur, Lucas and Best ('47) reported evidence of the replacement of choline by triethylcholine as a component of the liver lecithins, it appears that mere incorporation of choline-like compounds into the liver lecithins does not assure lipotropic activity.

The use of a more purified diet low in choline and possible choline precursors, vitamin B_{12} and folacin, more clearly illustrated the role of vitamin B_{12} and folacin in the utilization of choline precursors. The experiments herein reported indicate that vitamin B_{12} or folacin, or both, may function in the utilization of methionine or betaine for the synthesis of choline from aminoethanol or methylaminoethanol.

In vitro studies now in progress with surviving liver slices indicate that vitamin B_{12} is involved in the synthesis of choline from methylaminoethanol and methionine.

SUMMARY

1. The choline requirement of rats for the maintenance of normal liver fat is greatly reduced by supplementing the diet with vitamin B_{12} and folacin.

2. For lipotropism and protection against renal damage in rats, vitamin B_{12} and folacin increased the efficiency of betaine, DL-methionine, dimethylaminoethanol, and methylaminoethanol as replacements for dietary choline.

3. The triethylhomologue of choline fed to weanling rats is not capable of replacing choline as a lipotropic agent.

4. Vitamin B_{12} and folacin appear to be essential for the maximum utilization of methionine or betaine for the biological synthesis of choline from aminoethanol or methylaminoethanol.

ACKNOWLEDGMENTS

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EFFECT ON CHICK GROWTH OF AMINO ACID IMBALANCES IN DIETS CONTAINING LOW AND ADEQUATE LEVELS OF NIACIN AND PYRIDOXINE [1]

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ONE FIGURE

(Received for publication May 28, 1951)

It is now recognized that the niacin requirement of certain animals is influenced by the protein of the diet. When proteins or amino acid mixtures low in tryptophan are added to niacin-low diets, the growth rate is depressed; but if the diet contains an adequate level of niacin or tryptophan, the growth depression does not occur. The beneficial effect of tryptophan is apparently due to its conversion to niacin. The effect of additions of tryptophan-low proteins to niacin-low diets and the conversion of tryptophan to niacin has been reviewed by Krehl ('49).

Previous work indicates that amino acids vary in their ability to depress growth when added to niacin-low diets. The

[1] Scientific paper A328. Contribution 2293 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry).

Portions of the data given in this paper were presented at the meeting of the American Society of Biological Chemistry, Chicago, Illinois, 1947 (Fed. Proc., *6:* 257) and at the meeting of the Poultry Science Association, Guelph, Ontario, 1949 (Poultry Sci., *28:* 755).

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reasons for these differences and the cause for the growth depression are not completely understood. Hankes et al. ('49) have suggested that additions of threonine and phenylalanine to the diet of rats depress growth by accentuating the deficiency of tryptophan. Groschke and Briggs ('46) and Briggs et al. ('46) found that the glycine, alanine, and arginine present in gelatin were at least partly responsible for the growth-depressing action noted when this protein was added to niacin-low chick diets. Groschke et al. ('48) found that the growth-depressing action of zein could be duplicated by a combination of glutamic acid, leucine, alanine, proline, and phenylalanine.

In the studies described here, each of 16 amino acids was added to niacin-low diets at a uniform level to determine which were most responsible for the growth depression caused by tryptophan-low proteins. Similar studies were also made with pyridoxine-low diets. Experiments were carried out to determine whether the ability of the chick to convert an excess of some amino acids to other substances is impaired by a niacin deficiency. In addition to observations on growth rate, deficiency symptoms, and mortality, blood levels of amino nitrogen, nonprotein nitrogen, and glucose were determined.

EXPERIMENTAL PROCEDURE

The basal diet (112) had the following percentage composition: crude casein 18; gelatin 5; salts 1M (Briggs, '46) 6; soybean oil 4, DL-methionine 0.3; and glucose [5] 66.4 Each 100 gm of diet also contained the following amounts of vitamins (in milligrams): thiamine HCl 0.4; riboflavin 0.8; calcium pantothenate 2.0; pyridoxine HCl 0.6; folic acid 0.2; niacin 10; choline Cl 200; inositol 100; biotin 0.02, *p*-aminobenzoic acid 0.2; menadione 0.1; and alpha-tocopherol acetate, 0.5. Vitamins A and D_3 (1,200 U.S.P. units and 170 A.O.A.C. units, respectively) were given by dropper weekly. The nia-

[5] Cerelose.

cin-low diet, designated 112-N, contained no added niacin. The pyridoxine-low diet (112-P) contained 75 μg of added pyridoxine per 100 gm.

The level of each of the essential amino acids was calculated and the acids, except for arginine, were found to be present in amounts equal to or greater than the requirements suggested by Almquist ('47). Experimental evidence indicates that for a maximum growth rate there is a slight need for additional protein as well as the amino acid, arginine.

All of the amino acids and keto acids used in these experiments, except the threonine-allothreonine mixture and phenylpyruvic acid, were obtained through commercial channels. When keto or amino acids were added singly to the diet, a level of 4% was used in place of an equal amount of glucose.

In all experiments, day-old New Hampshire chicks of mixed sexes were used. They were the progeny of dams fed a complete breeder ration. At the beginning of each experiment the chicks were distributed into uniform groups and placed in electrically heated batteries with wire floors. Feed and water were supplied ad libitum. Weighings and other observations were made weekly. Each experiment was conducted for a period of 4 weeks.

At the termination of some of the experiments the blood levels of amino nitrogen, nonprotein nitrogen and glucose were determined. All blood was withdrawn by heart puncture after the birds had been fasted for approximately 12 hours. Blood from all birds within a group was pooled and the determinations made on a group sample. The protein-free blood filtrates were prepared and the nonprotein nitrogen level determined by the method of Folin and Wu ('19). Glucose was determined by the method of Benedict ('31) and amino nitrogen by the method of Danielson as modified by Hawk, Oser and Summerson ('47).

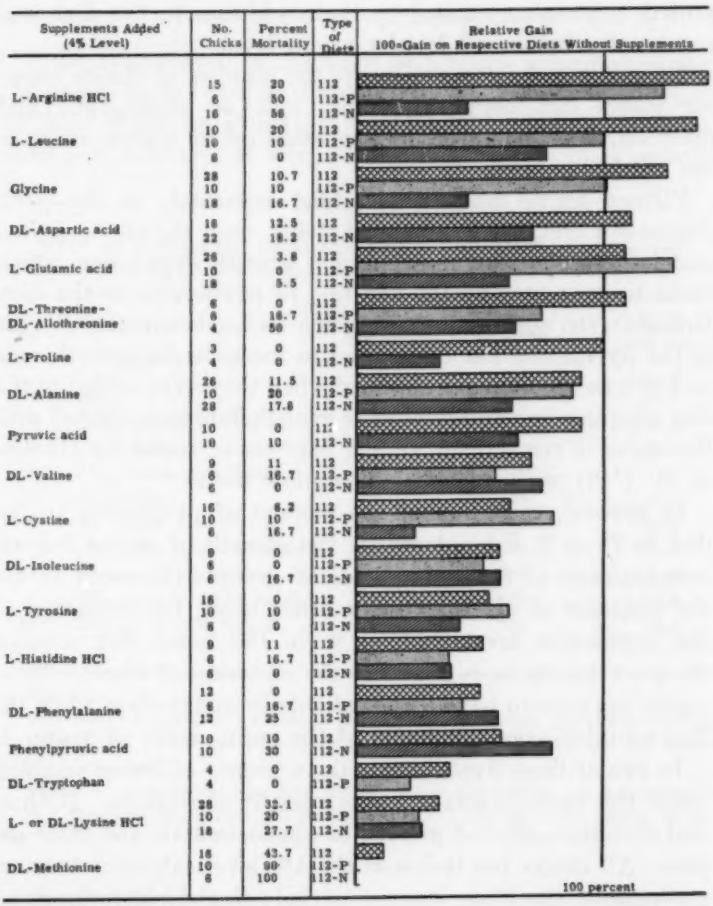
EXPERIMENTAL RESULTS

Results of feeding high levels of individual amino acids

The study of the effects of adding individual amino acids consisted of 7 experiments. Throughout the experiments there was good agreement as to the relative influence the addition of a particular amino acid had on chick growth. A summary of these experiments is given in figure 1.

In figure 1 the amino acids are listed in declining order of the growth which resulted from the addition of each at a level of 4% to diet 112, containing adequate levels of niacin and pyridoxine. Most of these amino acids also were added at the same level to the niacin-low (112-N) and the pyridoxine-low (112-P) diets. The length of each bar in figure 1 is proportional to the gain of the chicks fed the amino acid, expressed as a per cent of the gain of the chicks fed the same diet without the added amino acid. Thus, the gain of chicks fed the niacin-low diet containing an added amino acid is compared to that of chicks fed the niacin-low basal diet 112-N. The average gain of the chicks fed diets 112, 112-N, and 112-P was 196, 175, and 140 gm, respectively.

On the basis of their effect on growth, the amino acids can be divided into two groups. The first group, which will be referred to as group A, includes the amino acids arginine, leucine, glycine, aspartic acid, glutamic acid, threonine, proline, alanine, cystine, and probably histidine. The addition of these amino acids to diet 112 either increased the growth rate or did not decrease it as much as the other amino acids. Moreover, the addition of each of these amino acids to the niacin-low diet (112-N) resulted in a growth depression which was completely or partially overcome by the addition of niacin. The second group of amino acids, which will be referred to as group B, includes valine, isoleucine, tyrosine, phenylalanine, tryptophan, lysine, and methionine. These amino acids depressed growth more than the others, and the



* - diet 112 (niacin and pyridoxine adequate)
diet 112-P (pyridoxine - low)
diet 112-N (niacin - low)

Fig. 1 Summary of the effect on chick growth of individual amino and keto acids added to the diet at a level of 4%. The length of each bar is proportional to the gain of chicks fed the amino or keto acid supplement, expressed as a per cent of the gain of chicks fed the same diet without the added amino or keto acid. The average gains of chicks fed diets 112, 112-P and 112-N during 4 weeks were 196, 140, and 175 gm, respectively.

growth depression caused by their addition to the diet was not greatly influenced by the niacin level.

Throughout these experiments the number of chicks showing niacin deficiency symptoms, such as blacktongue and diarrhea, was increased by the addition of amino acids to the niacin-low diet.

Fifteen amino acids were added separately to the pyridoxine-low diet, 112-P, at a level of 4%. Only the DL-threonine-DL-allothreonine mixture caused a growth depression which could be overcome by the addition of pyridoxine to the diet. However, the addition of L-arginine HCl, L-leucine, or glycine to the pyridoxine-low diet failed to increase the growth rate as much as the increase obtained when they were added to the diet adequate in pyridoxine. The growth depression noted with threonine is reminiscent of the depression found by Hankes et al. ('48) with rats fed niacin-low diets.

In general, reduction of the amount of pyridoxine in the diet to 75 μg % did not reduce the growth of chicks fed excess amounts of the amino acids of group A as much as did the omission of niacin. On the other hand, the reduction of the pyridoxine level to 75 μg % in the basal diet reduced the growth rate more than did the omission of niacin. Thus, niacin appears to have a special role in metabolism when the diet contains excess amounts of the amino acids of group A.

In two of these experiments the L-isomer of lysine was fed, while the racemic mixture was used in the others. Both L- and DL-lysine affected growth to approximately the same degree. All chicks fed lysine at the 4% level exhibited tremors throughout the experimental period; the chicks were weak and the feathering very poor. Their condition resembled that of chicks fed a diet deficient in arginine.

A peculiar condition was noted in the chicks fed diet 112-N plus 4% added DL-alanine. These chicks tended to stand in a fixed position with little movement. The movements that were made were slow and deliberate. The condition appeared to be somewhat improved by the end of the 4-week period.

Studies with amino acid mixtures

Since it appeared in the work with single amino acids that the amino acids could be divided into two groups on the basis of their effects on growth, it was thought desirable to determine how mixtures of amino acids would affect chick growth.

TABLE 1

Effect of proteins and amino acid mixtures on growth of chicks fed diets with and without adequate niacin

GROUP NO.	ADDITION TO DIET 112-N	NO. CHICKS [1]	AVE WT. AT 4 WKS.	BLOOD AMINO NITROGEN
			gm	*mg %*
1	None	8	182	20.7
2	10 mg niacin per 100 gm	10	250	22.5
3	5% gelatin	9	180	20.7
4	5% gelatin plus niacin	9	288	18.4
5	5% casein	10	193	20.7
6	5% casein plus niacin	9	276	20.6
7	5% group A amino acids [2]	7	137	21.3
8	Same as 7 plus niacin	7	299	16.8
9	5% group B amino acids [3]	8	118	19.6
10	Same as 9 plus niacin	10	104	19.4

[1] Chicks remaining at the end of the experiment; all groups were started with 10 chicks.

[2] This mixture supplied L-arginine HCl 0.77%; threonine (mixture) 0.12%; L-glutamic acid 0.84%; DL-aspartic acid 0.51%; glycine 1.94%; and DL-alanine 0.82% in the diet.

[3] Amino acids were DL-lysine HCl 0.97%; L-tyrosine 0.90%; DL-phenylalanine 0.73%; DL-methionine 0.49%; DL-isoleucine 0.92%; and DL-valine 0.99% in the diet.

In the first experiment a mixture was used which contained 6 amino acids of group A in the same proportions and amounts as are supplied by approximately 85% gelatin. A second mixture was also used which contained 6 amino acids of group B in the same proportions and amounts as are supplied by 15% casein. Each of these mixtures was fed at a level of 5% of the diet. Results of this experiment are given in table 1.

Only a moderate deficiency of niacin was obtained, probably due to an unusually large amount present in the crude casein used. Nevertheless, the mixture of the amino acids of group A depressed growth when added to the niacin-low diet, and the depression was overcome by the addition of niacin to the diet. The mixture of amino acids of group B depressed growth when added to the diet either in the presence or

TABLE 2

Effect of mixtures of amino acids of groups A and B on growth of chicks fed diets with and without adequate niacin

GROUP NO.	ADDITION TO DIET 112-N	CHICKS STARTED	MORTALITY	AVE. WT., 4 WKS.
			%	*gm*
1	None	12	8.3	200
2	10 mg niacin per 100 gm	10	0	237
3	1% L-arginine HCl plus 1% DL-aspartic acid plus 1% glycine	15	13.3	134
4	1% DL-valine plus 1% DL-isoleucine plus 1% L-tyrosine	15	20.0	157
5	Same as 3 plus amino acids of 4	15	66.7	127
6	Same as 3 plus 1% L-glutamic acid plus 1% DL-alanine plus 1% L-histidine HCl	12	66.7	74
7	Same as 4 plus 1% DL-lysine HCl plus 1% DL-phenylalanine plus 1% DL-methionine	15	20.0	77
8	Same as 7 plus niacin	15	26.6	91

absence of added dietary niacin. Thus, the mixtures had the same effects on growth as were noted when the single amino acids of each group were added to the diet at a level of 4%. This was also found to be true in a second experiment.

In the second experiment, a water-washed casein was used, and all chicks were injected with 0.3 μg of crystalline vitamin B_{12} per week. Five different combinations of amino acids (see table 2) were fed chicks in the niacin-low diet (112-N). The addition of niacin to the diet containing 6 amino acids

of group B did not overcome the growth depression caused by the addition of these amino acids. The addition of three amino acids of group A and three of group B to the niacin-low diet (group 5, table 2) did not depress the growth rate as much as the 6 amino acids of either group fed at comparable levels (groups 6 and 7, table 2). The difference between group 5 and groups 6 and 7 is statistically significant. The effect of other combinations of amino acids is not known; however, these results suggest that additions of combinations of amino acids of groups A and B to niacin-low diets do not depress growth as much as additions of equal amounts of the amino acids of either group alone.

Results of determinations of nonprotein nitrogen, amino nitrogen, and glucose

There was no correlation between blood nonprotein nitrogen level and growth rate. The blood level of amino nitrogen was increased by the addition of each amino acid except DL-lysine HCl, DL-methionine, and L-arginine HCl. The inclusion of 5% additional gelatin in the diet also decreased the amino nitrogen level.

The chicks fed diets with 4% added methionine or lysine gained weight very slowly and consumed very little feed. It is believed that the low blood amino nitrogen level reflected only the general condition of the chicks. The decreased blood amino nitrogen level of chicks fed diets with added arginine or gelatin is believed to be a result of a more efficient utilization of the protein of the diet, since diet 112 was slightly deficient in arginine.

In two experiments in which L-arginine HCl was fed, blood filtrates from the chicks were tested for arginine by the method of Weber ('30). The addition of arginine to the niacin-low diet (112-N) or to the pyridoxine-low diet (112-P) increased the level of arginine in the blood. An equal or greater increase was observed when arginine was added to the diet ade-

quate in niacin and pyridoxine. There was no indication, therefore, that the addition of niacin or pyridoxine to the diet corrected this arginine imbalance.

A summary of the blood glucose levels found in the experiments wherein diet 112 or 112-N was used is given in table 3. Chicks fed diet 112-N (without added niacin) had a consistently lower fasting blood glucose level than chicks fed the same diet with added niacin. The mean difference for 6 experiments was 10 mg %. Chicks fed diet 112 (with niacin) had higher blood sugar levels when each of the 7 amino acids

TABLE 3

Influence of amino acids and a niacin deficiency on the fasting blood sugar level of the chick

GROUP NO.	ADDITIONS TO THE DIET	DIET 112 (with niacin)	DIET 112-N (without niacin)
		mg %	
1	None	172	162
2	4% L-arginine HCl	187	146
3	4% glycine	..	160
4	4% DL-aspartic acid	182	146
5	4% DL-glutamic acid	181	160
6	4% DL-alanine	178	150
7	4% DL-phenylalanine	196	..
8	4% DL-lysine HCl	176	186
9	4% DL-methionine	180	168

was added to the diet. However, the fasting blood glucose level was decreased when 5 of these amino acids, all of group A, were added to diet 112-N. In other experiments in which a complete practical-type starting ration was fed, increases were noted after feeding DL-methionine, glycine, L-leucine, DL-lysine HCl, L-cystine, L-histidine HCl, L-tyrosine, or L-glutamic acid. These differences in blood glucose level were not noted prior to fasting.

The results of the first experiments, in which excesses of single amino acids were added to the diet, suggested that niacin is needed to correct the amino acid imbalance produced. It seemed likely that an amino acid imbalance might

decrease the rate of protein synthesis and thus explain the reason for the growth depression. If this were true, a growth depression would be expected when the amino acids were added to a diet deficient in any vitamin required to correct the imbalance. However, decreasing the pyridoxine level of the diet caused a growth depression similar to that noted with niacin-low diets only with the threonine-allothreonine mixture. This made it doubtful that the effect of niacin in overcoming the growth depression (noted when certain amino acids were added to the niacin-low diet) could be attributed to an increased ability of the chick to correct the amino acid imbalance. Two other observations indicated that other factors were involved. First, when pyruvic acid was added to the diets with low and adequate levels of niacin, the growth of the chicks in each case was similar to that noted when alanine was added to the same diets. Second, there was no indication that niacin in the diet helped to decrease the excess level of arginine found in the blood of chicks fed this amino acid.

It is interesting to observe that most of the amino acids of group A are those which have been termed "glucogenic" by Mitchell ('46), while most of those in group B have been termed "ketogenic." However coincidental this may be, the division of amino acids into these two types has never been clearly defined, the classification depending upon the criteria used. In these experiments the growth depression caused by feeding the amino acids of group B does not appear to be related to their relative ability to form ketone bodies; nor does the growth depression caused by feeding amino acids of group A in niacin-low diets seem to be related to their relative ability to form glucose. While leucine is usually considered to be ketogenic, it is included in the group A amino acids.

Histidine depressed the growth of chicks more than the other amino acids of group A; however, the magnitude of the imbalance, in terms of the accepted normal requirement, was greater with histidine than with any other amino acid.

In these tests most of the amino acids of group B were fed in the DL-form. However, it was found that L-lysine HCl and DL-lysine HCl caused approximately the same growth depression. Also, phenylpyruvic acid depressed growth almost as much as DL-phenylalanine. In work with rats, Wretlind and Rose ('50) found that both isomers of methionine depressed growth if fed in excessive quantities, the L-isomer being the more active in this respect. It is believed, therefore, that the effects noted in these experiments with the racemic amino acids of the ketogenic group were a result of the combined actions of both isomers.

If it is assumed that the growth depression noted when the amino acids of group B were fed was not an effect of feeding the D-isomers, the growth depression resulting from the feeding of the mixture of group B amino acids (group 10, table 1) is very significant. These amino acids were fed in the same amounts which would have been supplied by 15% casein. Other experiments have shown that the addition of either 12 or 22% casein to a similar diet increases the growth rate. Apparently the amino acids of group B depress the growth rate of chicks only when they are not balanced in the diet by the other amino acids. These results indicate that the growth-depressing action of certain proteins, when added to niacin-low diets, depends not only on their relative lack of tryptophan but also upon the proportion of the protein made up by the amino acids of group A.

The effect on growth of adding L-arginine HCl, DL-alanine, glycine, DL-aspartic acid, and L-glutamic acid to chick diets with low or adequate levels of niacin appears to be related to their effects on blood glucose level. When these amino acids were added to diet 112 (niacin), the fasting blood glucose level was increased. This effect is similar to the "protein effect" which was first noted in rats by Mirski et al. ('38). Todd et al. ('47) found that rats fed extra glycine or alanine had higher blood glucose levels after a period of fasting than animals fed the same diet without the added amino acids. Rats fed leucine or glutamic acid did not show the

effect. Todd and Tolman ('49) expressed the belief that feeding excess glycine stimulates the general process of glyconeogenesis, probably by increasing adrenal cortical activity. This "protein effect" was not noted in adrenalectomized rats.

The question naturally arises as to why this effect is not noted when glycine, arginine, alanine, aspartic acid, or glutamic acid is added to a niacin-low diet. In other experiments chickens which had previously been fed a commercial-type ration maintained their blood glucose level above 160 mg % for 72 hours during fasting. It is generally believed that hormones secreted by the adrenal cortex stimulate gluconeogenesis, as these reactions are needed to maintain the blood sugar level during a fast. This suggests that the endocrine systems which control the blood sugar level do not function properly in chicks fed a niacin-low diet, and that the 5 amino acids mentioned aggravate this condition. Handler and Dann ('42) have already suggested that some of the symptoms of niacin deficiency in the dog are a result of a hypofunction of the adrenal cortex.

SUMMARY

Amino acid imbalances were produced in chick diets by the addition of 4% of single amino acids to diets low and adequate in niacin and pyridoxine in which the protein was furnished by 18% crude casein, 5% gelatin, and 0.3% DL-methionine. When L-arginine HCl, L-leucine, glycine, DL-aspartic acid, L-glutamic acid, or a DL-threonine-DL-allothreonine mixture was added to a diet adequate in niacin and pyridoxine, the growth rate of the chicks was not depressed. Additions of L-proline or DL-alanine decreased the growth rate slightly. Additions of DL-valine, L-cystine, DL-isoleucine, L-tyrosine, L-histidine HCl, DL-phenylalanine, DL-tryptophan, L- or DL-lysine HCl, or DL-methionine markedly decreased the growth rate. All amino acids depressed growth when added to a niacin-low diet; only DL-threonine-DL-allo-

threonine caused a growth depression when added to a pyridoxine-low diet, and this could be overcome by the addition of pyridoxine.

The amino acids were divided into two groups on the basis of their effects on growth. One group included valine, tyrosine, phenylalanine, isoleucine, tryptophan, lysine, and methionine. The addition of these amino acids or mixtures of these amino acids to the chick diet depressed growth more than the other amino acids, and the growth depression was not greatly influenced by the addition of niacin to the diet. The other amino acids when added to the niacin-low diet caused a growth depression which was partly or entirely overcome by the addition of niacin to the diet.

Additions of amino acids to a diet containing adequate niacin increased the fasting blood sugar level of chicks, but additions of L-arginine HCl, DL-alanine, DL-aspartic acid, L-glutamic acid, or glycine to a niacin-low diet decreased the fasting blood sugar level. The blood amino nitrogen level was increased by the addition of each amino acid except methionine, lysine, and arginine. Possible mechanisms by which the amino acids cause these effects are discussed.

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THE SYNTHESIS OF "BIOLOGICALLY LABILE" METHYL GROUPS IN THE GERM-FREE RAT

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The purpose of the present investigation was to conduct an experiment which would permit a decision as to whether the synthesis of "biologically labile" methyl groups in the rat could take place in the absence of intestinal bacteria.[3] The plan of the experiment followed one used previously (du Vigneaud, Simmonds, Chandler and Cohn, '45) in which the synthesis of methyl groups was shown to occur in normal rats by maintaining a level of approximately 3% D_2O in their body water for about three weeks and measuring the level of deuterium resulting in the methyl groups of the tissue choline. In the present study this technique has been applied to *germ-free* as well as nonsterile rats.

[1] The Cornell group wish to express their appreciation to the Lederle Laboratories Division, American Cyanamid Company, for a research grant which aided greatly in this investigation.

[2] Part of the work at LOBUND was carried out under a contract with the Office of Naval Research.

[3] An historical background to the question of the biological synthesis of labile methyl groups and a discussion of the possible metabolic implications of the present findings have been presented in a preliminary report of this investigation (du Vigneaud, Ressler and Rachele, '50). The present paper will therefore not concern itself in detail with these aspects of the study.

Animals of the LOBUND strain were maintained with D_2O in their drinking water under both germ-free and nonsterile conditions at LOBUND and, for comparative purposes, animals of the Rockland Farms strain were maintained at the Cornell Laboratory on the same dietary regimen as that used at LOBUND but under the usual laboratory conditions. The animals were sacrificed, the choline was isolated from the tissues and degraded to trimethylamine, and the creatine was isolated as creatinine potassium picrate and degraded to methylamine. If, under these conditions, deuterium were found in the methyl groups of the choline and creatine isolated from the germ-free rats, synthesis of the methyl group must have occurred in the tissues of the animals. It was extremely unlikely that a direct exchange reaction between the hydrogens of "biologically labile" methyl groups and the deuterium of the D_2O in the tissue fluids could have taken place to cause the appearance of deuterium in the methyl group (du Vigneaud, Cohn, Chandler, Schenck and Simmonds, '41; Keller, Rachele and du Vigneaud, '49).

EXPERIMENTAL

Procedures for animal experiments

Two male, germ-free rats reared by the technique of Reyniers et al. were maintained in individual metabolic compartments under germ-free conditions at LOBUND (Reyniers, Trexler and Ervin, '46). The procedure permitted the administration of food and water and the separate collection of urine and feces under sterile conditions. Sterilized drinking water containing 10 atom % deuterium was furnished for 4 days, followed by water containing 4 atom % deuterium for the rest of the experimental period. An average deuterium level in the body water of approximately 2.5 atom % during the experimental period resulted. Two animals were also maintained on D_2O under nonsterile conditions.

The composition of the diet (LOBUND diet 231) containing 25% casein is given in table 1. This diet contained no choline.

It had previously been shown [4] that germ-free rats grew satisfactorily when fed a similar diet containing no added vitamin B_{12} and only 5 mg of choline per 100 gm of diet. Data on food and water consumption were noted daily. Routine bacteriological tests on the germ-free animals were run each week and prior to termination of the experiment. The method, which has been described previously (Reyniers, Trexler, Er-

TABLE 1

Metabolism data on LOBUND rats

	CONTROL NO. I	CONTROL NO. II	GERM-FREE NO. 5	GERM-FREE NO. 6
Age, days	61	61	127	127
Weight change, gm	183–253	179–282	294–322	308–289
Days fed 10% D_2O	4	4	4	4
Days fed 4% D_2O	17 [1]	17 [1]	19	6
Food [2] intake, average gm/day	14.4	17.3	16.2	13.0
Water intake, average ml/day:				
10% D_2O	21.0	24.1	28.4	40.0
4% D_2O	20.7	23.7	26.3	41.5
Urine, average ml/day	8.1	11.2	13.8	22.0
Feces, average gm/day	1.3	1.4	3.9	2.5

[1] Three per cent D_2O was fed for the first 7 days of this period.

[2] The diet had the following composition: casein (Labco) 25 gm, cellophane 2 gm, corn starch 59.5 gm, salt mixture 6 gm, corn oil 7 gm; vitamins/100 gm of diet: vitamin A (esters) 800 I.U., vitamin D_3 100 I.U., vitamin C 200 mg, vitamin E 50 mg, vitamin K 10 mg, inositol 100 mg, thiamine hydrochloride 6 mg, riboflavin 3 mg, pyridoxine hydrochloride 2 mg, pyridoxamine hydrochloride 0.4 mg, calcium pantothenate 30 mg, nicotinamide 5 mg, nicotinic acid 5 mg, biotin 0.1 mg, folic acid 1 mg, *p*-aminobenzoic acid 5 mg, vitamin B_{12} 0.01 mg.

vin, Wagner, Luckey and Gordon, '49), consisted of microscopic and cultural examination of samples of excreta, food, water, and swabbings from the skin, body orifices, cage interior, glove surfaces, and equipment. All tests were negative throughout the experiment.

The data on the LOBUND rats are summarized in table 1. The animals appeared healthy during the experiment except

[4] Luckey, T. D., and Reyniers, J. A., unpublished observation.

for a few hours during which the germ-free animals became overheated. The germ-free rats, which were older than the control rats, did not gain as much weight as the latter. Both germ-free rats showed every criterion of health at autopsy. Even the ceca (often enlarged in germ-free animals) were comparable to those of the control rats.

The germ-free animals were sacrificed within the germ-free environment, samples were taken for histological examination, the intestinal contents were removed separately, and the carcasses were cut into chunks and taken from the cage. The tissues were ground in alcohol under aseptic conditions and shipped, along with the sterile urine and feces, by air express to New York. The two animals fed D_2O and the sterilized diet but maintained under nonsterile conditions at LOBUND were processed in the same manner as that used for the germ-free animals. On arrival in New York the tissue samples were extracted continuously in a Soxhlet extractor with 95% ethanol overnight.

The animals maintained at the Cornell Laboratories on the LOBUND diet were furnished with D_2O in a manner similar to that used for the LOBUND animals. To eliminate any possible difference in source of dietary material, the experimental diet was shipped from LOBUND by air express to New York. Two of the animals at Cornell received an additional supplement of vitamin B_{12} in the diet, as is indicated in table 2. The animals were sacrificed after the indicated time periods. After the contents of the gastrointestinal tract had been washed out, the tissues of the rats were frozen with powdered dry ice and ground twice in a fine meat chopper. The ground tissue was then extracted continuously in a Soxhlet extractor with 95% ethanol overnight.

Urine specimens were collected from all of the animals during the entire experimental period. The average deuterium concentration of the urine collected from each animal was used to reflect the isotope concentration of the body fluid during the experimental period.

Isolation of choline and creatine

Tissue choline and creatine were isolated essentially by the procedure described by du Vigneaud and co-workers ('41) with certain modifications. Each of the alcoholic extracts described in the preceding section was evaporated under reduced pressure and the residue was suspended in about 150 ml of water and extracted several times with ether. The aqueous layer was made approximately 1 *N* acid with HCl and heated under reflux for two to three hours. The resulting solution was concentrated *in vacuo* to dryness, taken up in 10 ml of water, and made approximately 0.1 *N* with NaOH. It was usually necessary to filter the solution after it had been rendered basic. The alkaline filtrate was treated with a saturated solution of ammonium reineckate and cooled for several hours. The solution containing the suspended choline reineckate was centrifuged. The precipitate was washed with cold water and combined with that collected as described below from the ether extract obtained from the original alcohol concentrate. The combined aqueous supernatant and washings were saved for subsequent isolation of the tissue creatine in the form of creatinine potassium picrate.

The ether extract was freed from solvent and the residue was heated under reflux for three hours in a suspension of 5 gm of $Ba(OH)_2$ and 100 ml of water per 150-gm rat. The solution was then acidified with concentrated HCl while still warm and allowed to stand overnight, by which time the barium salts of the fatty acids were decomposed, and the fatty acids were extracted with ether. The aqueous layer was concentrated *in vacuo* to dryness and the residue was triturated well with absolute ethanol. The alcoholic extract was similarly concentrated to dryness, and the residue was taken up in 40 to 50 ml of water and made 0.1 *N* alkaline with NaOH. To the filtered solution a saturated ammonium reineckate solution was added until all the choline had been precipitated. After having stood several hours in the cold, the solution and suspended precipitate were centrifuged and the collected

precipitate was washed once with cold water, alcohol, and ether. The material was combined with the choline reineckate which was derived from the aqueous layer obtained from the original alcoholic extract.

The combined choline reineckate precipitates were dissolved in the minimum amount of acetone-water mixture (1:1) and treated with a saturated solution of silver sulfate. The pink

TABLE 2

Isolation of choline and creatine from germ-free and nonsterile rats

RAT NO.		EXPERIMENTAL PERIOD	CHANGE IN BODY WEIGHT	CHOLINE CHLOROPLATINATE [1]		TRIMETHYLAMINE CHLOROPLATINATE [2]	CREATININE POTASSIUM PICRATE	METHYLAMINE CHLOROPLATINATE [3]
		days	*gm*	*mg*	% *Pt*	% *Pt*	*Jaffe*	% *Pt*
CUMC [4]	713 ♀	19	97–126	210	31.3	36.8	100	...
CUMC [5]	710 ♂	21	119–237	469	31.4	37.1	...	...
CUMC [5]	707 ♂	21	109–186	429	31.7	36.7	102	...
CUMC	708 ♂	21	103–186	419	32.2	36.7	...	...
CUMC	709 ♂	21	105–225	462	31.2	36.8	101	41.4
LOBUND	I ♂	21	183–253	533	31.4	36.9	101	41.4
LOBUND	II ♂	21	180–282	579	31.2	37.0	101	41.6
LOBUND (germ-free)	5 ♂	23	294–322	661	31.4	36.8	98	41.6
LOBUND (germ-free)	6 ♂	10 [6]	308–289	632	31.4	37.2	101	41.5

[1] Theoretical platinum content: 31.7.
[2] Theoretical platinum content: 37.0.
[3] Theoretical platinum content: 41.4.
[4] CUMC — Cornell University Medical College, Rockland Farms strain.
[5] Received additional 1.5 mg vitamin B_{12} daily.
[6] Died from overheating.

silver reineckate precipitate was removed and washed with water by centrifugation. After the supernatant had been heated to boiling, it was treated dropwise with 10% $BaCl_2$ and filtered. The filtrate, practically free of inorganic material, was made slightly basic to litmus and concentrated *in vacuo* almost to dryness to remove ammonia. It was then acidified with HCl and taken completely to dryness. The

residue was extracted several times with a small volume of absolute alcohol. The combined extracts were filtered and treated with a solution of platinum chloride in absolute ethanol. The resulting precipitate of choline chloroplatinate was collected by centrifugation, washed twice with absolute ethanol and dried. It was then recrystallized from hot water.

For the isolation of creatinine, the filtrate and washings from the choline reineckate obtained from the aqueous extract of the original alcohol concentrate were treated with a concentrated solution of lead acetate to remove the reineckate ion as its lead salt. The latter was separated by centrifugation and washed once with water. The supernatant and washings were combined, made alkaline and again filtered. The precipitate was washed and the washings were added to the filtrate. The combined solutions were acidified to Congo red with concentrated HCl and treated with H_2S to remove lead. The precipitate was collected and washed and the washings and filtrate were combined. This solution was concentrated to dryness *in vacuo*, and the residue was taken up in water and diluted to a volume of 25 ml. The creatinine content of this solution was determined colorimetrically on an aliquot by the Jaffe reaction with the use of the Klett-Summerson photoelectric colorimeter and a green filter (no. 54) against a standard aqueous solution of creatinine zinc chloride. With the quantity of creatinine thus obtained, creatinine potassium picrate was prepared as follows: 4.05 mg of picric acid per milligram of creatinine, 2.4 mg of picric acid per milliliter of solution, and 0.12 mg of KOH per milligram of picric acid were added to the solution containing the creatinine after it had been neutralized with aqueous KOH until it was just slightly acid to Congo red. The resulting mixture was warmed in a boiling water bath until all the picric acid had dissolved. The solution was allowed to cool slowly at room temperature and then overnight in the refrigerator. The precipitate was separated from the supernatant by centrifugation. Usually it was necessary to recrystallize the precipitate twice from a solution of 0.2% potassium picrate, followed by at least two

recrystallizations from distilled water. Smaller losses were encountered when the product was allowed to cool at 5°C. for about 6 hours between recrystallizations. The creatinine potassium picrate was then dried *in vacuo* at 70° and analyzed colorimetrically by the Jaffe reaction.

Degradation procedures

The permanganate oxidation used for the degradation of choline followed essentially the procedure described by Lintzel and Fomin ('31). One hundred and seventy milligrams of choline chloroplatinate and 4 ml of saturated NaOH were placed in a Claisen flask through which a rapid stream of water vapor from a steam generator was passed. A 250-ml round-bottomed ground-jointed flask containing 6 ml of 0.1 *N* HCl served as a trap for the liberated trimethylamine. When the contents of the Claisen flask had been heated to boiling in this way, 15 ml of a 3.2% solution of potassium permanganate were added in very small portions while the mixture was steam distilled vigorously. Each addition was characterized by an initially green color followed by the precipitation of MnO_2. Addition of permanganate solution was terminated upon the appearance of a permanent purple color throughout the solution, after which steam distillation was continued for an additional 15 minutes. The contents of the acid trap were then evaporated to dryness under reduced pressure and the residue was dissolved in a small quantity of absolute ethanol. Trimethylamine hydrochloride was precipitated as the chloroplatinate from this alcoholic solution by the addition of an excess of platinum chloride in absolute ethanol. The precipitate was washed several times by suspension in ethanol and centrifugation. Recrystallization of the salt from a minimum volume of ethanol-water yielded approximately 100 mg of bright orange prisms of trimethylamine chloroplatinate.

Two hundred and fifty milligrams of creatinine potassium picrate were suspended in 15 ml of 10% HCl and shaken repeatedly with ether. The acid solution was then concentrated

in vacuo to dryness and the white residue was taken up in 10 ml of water. The solution was rendered neutral to litmus with dilute NaOH and then heated under reflux for 48 hours with 220 mg of $Ba(OH)_2$. The solution was then separated from the precipitated $BaCO_3$ by filtration into a 100-ml Claisen flask. Three milliliters of 20% NaOH solution were added to the filtrate, which was then oxidized dropwise with 7 ml of saturated permanganate solution in the manner described for the conversion of choline to trimethylamine. The dilute HCl solution containing the methylamine was concentrated *in vacuo* to dryness and the residue was dissolved in hot absolute ethanol and precipitated with an excess of platinum chloride. A fractional crystallization from dilute HCl followed by a recrystallization of the more soluble material resulted in yellow-gold prisms of methylamine chloroplatinate in approximately 30% yield. The fractional crystallization was applied to effect a separation from ammonium chloroplatinate formed by the oxidation of any unhydrolyzed creatinine or of urea, methyl hydantoin and methyl hydantoic acid, which were presumably formed along with the sarcosine during the hydrolysis of creatinine with $Ba(OH)_2$. These hydrolysis products have been described by Gaebler ('26).

In table 2 may be found the analytical data on the creatinine potassium picrates and choline chloroplatinates isolated, and the methylamine and trimethylamine chloroplatinates, respectively, obtained from them. The deuterium content of the methyl groups of the choline and creatine and the ratio of these values to the isotope concentration of the body water during the experimental period are presented in table 3.

Attempted isolation of choline from feces of germ-free and nonsterile rats

Inasmuch as the animals were maintained on a choline-free diet during the experimental period, it was considered of interest to investigate whether choline was present in the feces of the germ-free and nonsterile rats. Jacobi, Baumann

and Meek ('41) were unable to detect any choline in a 7-day fecal specimen from animals growing on a choline-free diet.

The combined specimens of the feces of nonsterile LOBUND rats I and II (wet weight, 55 gm, collected over a 42-day period) and those of LOBUND germ-free rats 5 and 6 (wet weight, 110 gm, produced and collected under sterile conditions

TABLE 3

Deuterium content of methyl groups of choline and creatine

RAT NO.		DEUTERIUM CONTENT				DEUTERIUM CONTENT		
		Body water (A)[1]	Trimethylamine chloroplatinate (B)[2]	Methyl group of choline (C)[3]	$\frac{C}{A} \times 100$	Methyl amine chloroplatinate (E)[2]	Methyl group of creatine (F)[4]	$\frac{F}{A} \times 100$
		atom % excess	*atom % excess*	*atom % excess*		*atom % excess*	*atom % excess*	
CUMC[5]	713 ♀	3.10	0.17	0.19	6.1	...	...	...
CUMC	710 ♂	3.29	0.23	0.26	7.8	...	...	...
CUMC	707 ♂	3.08	0.19	0.21	6.9	...	...	...
CUMC	708 ♂	2.52	0.19	0.21	8.4	...	...	...
CUMC	709 ♂	3.01	0.21	0.23	7.8	0.14	0.29	9.6
LOBUND	I ♂	2.20	0.19	0.21	9.6	0.15	0.31	13.9
LOBUND	II ♂	2.27	0.18	0.20	8.8	0.11	0.22	9.5
LOBUND (germ-free)	5 ♂	2.44	0.14	0.16	6.4	0.09	0.17	7.0
LOBUND (germ-free)	6 ♂	2.99	0.09	0.10	3.3	0.06	0.12	4.0

[1] Determined by falling-drop procedure (error = ± 0.06).

[2] Determined with mass spectrometer (error = ± 0.02).

[3] Calculated value: $C = \frac{10}{9} B$.

[4] Calculated value: $F = 2 E$.

[5] See footnote 4, table 2.

over a 32-day period) were each dried, ground, extracted with 95% ethanol, and processed for choline according to the procedure described for the isolation of tissue choline. Less than 1 mg of a reineckate was obtained in the case of rats I and II, and in the case of the germ-free rats 5 and 6 there was no discernible precipitate.

Evidence for non-lability of hydrogens in methyl groups of choline and creatine under conditions of isolation and degradation

The following experiments have been used as evidence that the hydrogen atoms in the methyl groups of choline are not labile under the conditions used for the isolation and degradation of body choline. The results render it highly improbable that the gain of deuterium into the choline methyl groups in rats whose body water contained D_2O could have occurred by simple exchange between the hydrogens of the methyl groups of the body choline and the deuterium-enriched body water in the tissues of the animal.

In the study of transmethylation (Keller, Rachele and du Vigneaud, '49) in which methionine labeled with both C^{14} and deuterium in the methyl group was administered to a rat, the choline and creatine isolated from the tissues contained the same ratio of deuterium to C^{14} per methyl group as the administered methionine. This ratio was calculated on the assumption that all the isotope was confined to the methyl group. This assumption has recently been verified in a comparable experiment by degrading the choline to trimethylamine and obtaining the deuterium-C^{14} ratio in the latter (du Vigneaud, Verly, Wilson, Rachele, Ressler and Kinney, '51).

It was found in an earlier study (du Vigneaud and coworkers, '41) that the trimethylamine obtained from the permanganate degradation of the body choline isolated from rats fed methionine labeled with deuterium in the methyl group had the same deuterium content as the isolated choline. In addition to confining to the methyl group the deuterium present in the tissue choline, this experiment showed that no labilization of hydrogen occurred in the conversion of choline to trimethylamine. As further assurance that no deuterium would be lost from the methyl group during the conversion of choline to trimethylamine in our hands, this degradation was carried out on a sample of synthetic choline chloro-

platinate containing deuterium in the methyl group. Analysis showed 18.6 ± 0.3 atom % excess deuterium. After dilution with nonisotopic material to contain 3.15 ± 0.05 atom % excess deuterium or 0.88 ± 0.01 atoms of deuterium, it was degraded and converted by the method already described to trimethylamine chloroplatinate. The latter compound contained 4.5 ± 0.1 atom % excess deuterium or 0.90 ± 0.2 atoms of deuterium, and thus the isotope contents of the choline and the trimethylamine were the same within the limits of experimental error.

Similarly, the degradation of a sample of creatinine potassium picrate labeled in the methyl group with deuterium in known concentration was found to yield methylamine chloroplatinate of the same isotope concentration in the methyl group. Creatinine potassium picrate, isolated from the tissues of a rat fed methionine labeled in the methyl group with deuterium, contained 1.97 ± 0.07 atom % excess deuterium or 0.24 ± 0.01 atoms of deuterium. On degradation it gave methylamine chloroplatinate with a deuterium concentration of 4.0 ± 0.1 atom % excess or 0.24 ± 0.01 atoms of deuterium. Therefore, no labilization of hydrogen in this degradation appeared to be indicated.

Deuterium determinations

The isotope concentration of the body water was obtained by the use of the falling-drop procedure on a sample of the pooled urine specimens after suitable purification. After being subjected to a slow distillation with a current of oxygen through a furnace kept at 750°C., the water was further treated essentially as described by Keston, Rittenberg and Schoenheimer ('37), with the modifications given by Cohn ('46).

To determine the deuterium content of the trimethylamine and methylamine chloroplatinates obtained by degradation of the isolated tissue choline and creatine, 5 to 9 mg of the compounds were burned in a stream of oxygen. The resultant D_2O-H_2O mixtures were reduced to a mixture of deuterium

and hydrogen with zinc as described briefly by Sprinson and Rittenberg ('48). Construction and procedural details for the reduction apparatus were obtained from Dr. Rittenberg.[5] The gaseous mixtures were then introduced into the mass spectrometer and the ratios were determined as described by Washburn ('48).

DISCUSSION

From the data presented it is obvious that methyl groups had been synthesized by the germ-free animals. The enrichment of deuterium in the methyl groups of the choline isolated from the LOBUND germ-free rat after 23 days was 6.4% of that in the body water, and in the 10-day experiment involving the rat under germ-free conditions a value of 3.3% was obtained, an amount that agrees quite well with that of the longer experiment. A somewhat higher value was obtained in the LOBUND animals under nonsterile conditions, but this may not be a significant difference. While it is possible that additional synthesis by intestinal bacteria of labile methyl groups may have taken place in the case of the nonsterile animals, it may be noted that the germ-free animals were older and did not grow as much tissue during the experiment. Of ultimate importance, of course, is the fact that there was an enrichment of deuterium in the methyl groups of choline and creatine in the germ-free animals. It may be seen that the concentrations of deuterium in the methyl group of tissue creatine roughly paralleled those in the choline methyl group. It is also notable that the strain of animals maintained at Cornell on the LOBUND diet synthesized methyl groups to about the same degree as the LOBUND animals. The additional amount of vitamin B_{12} administered to two of the Cornell animals did not increase the degree of methyl synthesis.

Although it is choline that has been isolated to demonstrate the synthesis of the labile methyl group in the tissues, this does not imply that it is in the actual synthesis of this compound

[5] Rittenberg, D., private communication.

that a new methyl group is created. It could well be that the new methyl group is formed during the synthesis of methionine from homocysteine, and then through transmethylation the methyl group eventually arrives in the choline.

Demonstration of the presence of deuterium in the methyl group of creatine offers additional evidence of the synthesis of the labile methyl group. Although the methyl group of creatine is not labile in itself, it has been amply proved that the methyl group of creatine can be derived from a labile methyl compound by transmethylation. Again, the presence of deuterium in the creatine methyl group does not imply that in the actual synthesis of creatine a new methyl group has been created. The type of experimental procedure employed does not allow a decision on this point in the case of choline or creatine.

It would seem to us unlikely that the body possesses parallel mechanisms for the synthesis of each of the various "biologically labile" methyl compounds. It would seem more probable that the synthesis of the methyl group may be involved in the formation of one or a limited number of labile methyl compounds, and that this methyl group is eventually utilized by transmethylation for the synthesis of other N- and S-methyl compounds.

SUMMARY

A metabolic experiment with germ-free rats fed deuterium oxide has demonstrated that animals living in the complete absence of microorganisms are capable of synthesizing the labile methyl group, as shown by the presence of deuterium in the methyl group of certain compounds such as tissue choline and creatine. The degree of synthesis was comparable to that observed in control multicontaminated rats.

The methods of isolation of choline and creatine from tissues, the degradation of these compounds, and the quantitative isotope determinations are presented.

An attempt to isolate choline from the feces of both the germ-free and the nonsterile animals is also described.

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THE RELATION OF VITAMIN B_{12} DEFICIENCY TO FERTILITY OF THE FEMALE AND BIRTH WEIGHT OF THE YOUNG IN RATS FED PURIFIED CASEIN RATIONS

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Improved performance with regard to several aspects of reproduction and lactation in rats has been briefly reported from this laboratory when a 15 U./ml antipernicious anemia (APA) liver extract or, later, crystalline vitamin B_{12} was fed to the mothers as a separate supplement to B_{12}-deficient rations containing alcohol-extracted casein as the primary or sole source of protein (Hartman et al., '46; Cary and Hartman, '48; Dryden, Hartman and Cary, '49; Hartman, Dryden and Cary, '49). The present paper concerns the results observed on these rations with regard to comparative numbers of pregnancies and resorptions, litter size and birth weights of the young, and the extent to which vitamin B_{12} supplementation of the mothers prevented or overcame any defects observed in these respects.

Mapson ('32) observed a smaller litter size but no difference in birth weights when he compared litters from mothers on his basal ration containing "light white casein" to those from mothers fed the same ration plus a supplement of fresh liver. Bahrs ('33) also reported an increased litter size when fresh liver was fed as a supplement to a ration con-

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taining unpurified casein. Smith and Seegers ('34a, b) observed increased litter size and birth weight when the alcohol-extracted liver in their ration was replaced by dried whole liver or when the ration was supplemented with fresh liver. Schultze ('50), using a ration containing a commercial soybean protein preparation, found that the incorporation of a liver extract in the ration decreased the average number of resorption sites and increased the average litter size for females examined on the 21st day of pregnancy; however, neither of these two differences was statistically significant and no difference was observed in the average weight of the 21-day-old fetus. Lepkovsky et al. ('51) observed that, as rats were maintained through several pregnancies on a B_{12}-deficient ration composed primarily of cereal grains and vegetable seed products, there occurred a progressive increase in the number of resorptions and decrease in the number of conceptions, in litter size and in birth weight of the young; however, it is not clear from the data presented that vitamin B_{12} would overcome all these defects.

Improved performance resulting from the use of liver or liver extracts is not, of course, necessarily due in whole or in part to their vitamin B_{12} content. The experiments reported in this paper include B_{12}-deficient rations supplemented in some cases with an APA liver extract and in others with crystalline vitamin B_{12}.

EXPERIMENTAL PROCEDURE

The rats used in these experiments were stock colony[2] mothers that had given birth to one or more litters prior to being placed on the various experimental rations. Each mother, together with her litter, was placed at parturition or a day or so thereafter upon the experimental diet and, after the young were weaned, was continued on the experi-

[2] Composition of stock ration: yellow corn meal 69.5; linseed oil meal 14.0; meat scrap 9.0; casein (commercial) 4.0; alfalfa meal 2.0; bone meal 2.0; sodium chloride 0.5. Supplements of lettuce and carrots were fed once a week.

mental ration for a number of successive matings. A rest period of about one week was allowed between weaning and mating. Each mother was kept in an individual cage, on a raised screen floor during the mating and resting periods but on wood shavings at all other times.

Stock males were used for all matings except those in the F_1, F_2 and F_3 generations of the 4th experiment. A male was kept in the cage with a female until some indication of possible pregnancy was observed or until the end of a 14-day period, when it was replaced by another male. Frequent observations were made for vaginal plugs, placental blood signs and changes in weight. The appearance of a placental blood sign was taken as a positive sign of pregnancy and failure to litter thereafter was considered a resorption.

In the first three experiments and in the 5th experiment litters were reduced to a total of 6 at the age of one week; in the 4th experiment they were reduced to 6 at birth, a procedure that was likewise followed in the stock colony. The young retained were weaned at 25 days of age.

A number of B_{12}-deficient rations have been used in these experiments. Their composition is described in table 1. All of them are essentially modifications of ration 45. Such modifications include the addition of various amounts of pure vitamins, methionine, lactose and succinylsulfathiazole. The primary modification has been the replacement of yeast by pure B vitamins in some rations; the other modifications did not appear to have any noticeable effects on the aspects of reproduction considered in this paper and, accordingly, will not be further discussed, although the groups on these modified rations are shown separately in the tables. All rations and distilled water were supplied ad libitum. Supplements of 15 U./ml APA liver extract [3] or crystalline vitamin B_{12} [4] were administered orally, either daily or three times per week.

[3] Lederle.

[4] The crystalline vitamin B_{12} was kindly supplied by Dr. D. F. Green and Dr. M. A. Schooley, Merck and Co., Inc., Rahway, N. J.

TABLE 1

Composition of rations[1]

	RATION NUMBER							
	45	195	196	197	301	303	443	445
	%	%	%	%	%	%	%	%
Dextrin[2]	55.50	59.85	59.20	55.28	45.32	59.32	53.43	29.93
Lactose (U.S.P. XIII)	..	..	..	..	15.00	..	1.50	15.00
Casein (10 × hot alcohol-ext.)[3]	20.00	25.00	25.00	20.00	25.00	25.00	30.00	20.00
DL-methionine	..	0.20	0.40	0.20	..	..	0.20	0.20
Yeast, dried brewers'[4]	10.00	..	..	10.00	..	..	..	20.00
Salts (Hawk and Oser, '31)	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50
Succinylsulfathiazole	..	..	..	..	..	1.00	..	..
Cottonseed oil	9.85	9.85	9.85	9.85	9.85	9.85	9.85	9.85
Fish liver oil[5]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Vitamins added	..	0.46	0.91	0.03	0.18	0.18	0.37	0.37
Vitamins (mg/100 gm ration)								
Thiamine hydrochloride	..	1.6	3.2	..	0.8	0.8	1.6	1.6
Riboflavin	..	1.6	3.2	..	0.8	0.8	1.6	1.6
Pyridoxine hydrochloride	..	1.6	3.2	..	0.8	0.8	1.6	1.6
Calcium pantothenate	..	20.0	40.0	..	5.0	5.0	10.0	10.0
Choline chloride	..	240.0	480.0	..	120.0	120.0	240.0	240.0
Nicotinic acid	..	20.0	40.0	..	5.0	5.0	10.0	10.0
Inositol	..	20.0	40.0	..	5.0	5.0	10.0	10.0
Para-aminobenzoic acid	..	120.0	240.0	..	30.0	30.0	60.0	60.0
Biotin	..	0.01	0.02	..	0.01	0.01	0.02	0.02
Pteroylglutamic acid[6]	..	0.20	0.40	..	0.10	0.10	0.20	0.20
Ascorbic acid	..	10.0	20.0	10.0	4.86	4.86	10.0	10.0
Alpha-tocopherol acetate	..	20.0	40.0	20.0	9.72	9.72	20.0	20.0
2-methyl,1,4-naphthoquinone	..	0.5	1.0	0.5	0.25	0.25	0.5	0.5

[1] Ration 47 was the same as ration 45 and ration 194 as 197, except that in each case 10% dextrin was replaced by an additional 10% of yeast. Rations 192, 193, 300 and 302 were the same as rations 195, 196, 301 and 303, respectively, except that in each case 5% dextrin was replaced by an additional 5% of casein. Rations 47, 192, 193, 194, 300 and 302 were fed to the mothers during the lactation period; rations 45, 195, 196, 197, 301 and 303, respectively, were fed at all other times. Diets 443 and 445, where used, were fed at all times.

[2] "Amidex," a dextrinized cornstarch manufactured by the Corn Products Refining Co., New York, N. Y.

[3] For preparation of casein, see Hartman, Dryden and Cary ('51).

[4] Strain G, Anheuser-Busch Co., St. Louis, Mo.

[5] "Navitol with Viosterol (concentrated oleovitamin A and D) U.S.P.," E. R. Squibb and Sons, New York, N. Y. According to manufacturer, contained, per gram, 65,000 I.U. of vitamin A and 13,000 I.U. of vitamin D.

[6] Kindly supplied by Dr. E. L. R. Stokstad and Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

In the first and second experiments, the performance of mothers placed on the vitamin B_{12}-deficient rations was followed for a few successive matings. Then some of these mothers who failed to raise any of their young, or at least (in experiment 2) any of the young from their last litter, were given a supplement in experiment 1 of 15 U./ml APA liver extract (0.10 ml/day) and in experiment 2 of crystalline vitamin B_{12} (1 μg/day).

In the third experiment, primiparous stock colony mothers were put at parturition either on a vitamin B_{12}-deficient ration or on the same ration plus a supplement of 1 μg/day of crystalline B_{12}. The 5th experiment was carried out similarly, except that the mothers used were not always primiparous and the dose of crystalline vitamin B_{12} administered was 2 μg/day.

In the 4th experiment, 6 stock colony mothers were placed at parturition on a B_{12}-deficient ration. Some of the young (F_1 generation) that resulted from matings subsequent to the transfer to the B_{12}-deficient ration were maintained on a similar ration after weaning, while others were put at 28 days of age on the same ration plus a supplement of 15 U./ml liver extract. These two groups were carried through the F_2 and F_3 generations, every mother receiving the same diet as its parents. In the F_1, F_2 and F_3 generations, B_{12}-deficient males were mated to B_{12}-deficient females and B_{12}-supplemented males to B_{12}-supplemented females.

RESULTS AND DISCUSSION

Conceptions and resorptions

Comparisons of the relative numbers of conceptions and resorptions on the experimental rations are shown in table 2. Stock colony figures are given in addition, to serve as a standard of what could normally be expected of these rats.

It appeared conceivable that our experimental rations might be somewhat low in vitamin E, inasmuch as all of them contained ferric citrate and it is recognized that another ferric salt (ferric chloride) will cause destruction of vitamin E in

TABLE 2

Comparison of relative numbers of conceptions and resorptions on vitamin B_{12}-supplemented and unsupplemented B_{12}-deficient rations

EXPT.[1]	RATIONS	NO. OF FEMALES		PROPORTION OF:			
				Matings resulting in pregnancies		Pregnancies failing to result in litters	
		No B_{12}	B_{12}	No B_{12}	B_{12}	No B_{12}	B_{12}
				%	%	%	%
Average of expts. 1 and 2:				87	68	14	31
3[2]	192–195	10	10	70	72	10	38
3	300–301	5	5	60	73	22	27
3	302–303	5	5	93	67	29	20
3	194–197	10	10	73	80	18	17
4-F_1	45–47	12	10	83	84	18	17
4-F_2	45–47	21	13	70	71	11	20
4-F_3	45–47	14	3	81	93	11	14
5	192–195	5	7	80	57	12	12
5	443	7	6	93	83	0	10
Average of expts. 3, 4 and 5:				78	76	15	19
Stock[3]				79		11	
Average of all yeast-free groups:				82	72	13	22
Average of all yeast groups:				82	73	16	27
Vitamin E comparisons:[4]							
α-tocopherol acetate incorporated in ration				72		16	
3	All	51					
α-tocopherol acetate (3 mg/day) fed separately				69		27	

[1] Within each experiment, except 1 and 2, the comparisons are based on an equal number of matings for each rat: two matings in experiment 5, 5 in experiment 4 and three in experiment 3 (except for one animal on B_{12}-supplemented rations 192–195, which was mated only twice).

[2] In two rats on rations 192–195 without B_{12}, three on rations 192–195 plus B_{12} and one on rations 302–303 plus B_{12}, an obstruction of the Fallopian tubes (salpingitis) was found at autopsy.

[3] Figures based on 9,982 colony matings over a 5-year period.

[4] Groups on B_{12}-deficient and B_{12}-supplemented rations have been combined for this comparison inasmuch as there was no real difference in response.

rations under certain conditions. Accordingly, during the latter part of experiment 3, the alpha-tocopherol acetate was omitted from the rations and fed as a separate supplement. Comparisons are shown in table 2 for an equal number of matings before and after this change was made. No improvement in performance occurred in any respect. Furthermore, histological examinations of uterine sections of some of these animals that had resorbed their young failed to show the characteristic pigmentation associated with vitamin E deficiency (Mason and Emmel, '45).[5]

As can be seen from table 2, there did not appear to be any real differences attributable to the presence or absence of yeast in the rations.

In experiments 1 and 2, where the reproductive performance of the same rats was followed first on the B_{12}-deficient and later on the B_{12}-supplemented rations, there occurred in most cases a decrease in conceptions and an increase in resorptions when the supplemented rations were fed. Since these results could at least in part be accounted for by the more advanced age of these rats when they were fed the B_{12}-supplemented rations, a better comparison can be obtained from experiments 3, 4 and 5, inasmuch as in these experiments both B_{12}-supplemented and B_{12}-deficient groups were placed on the respective diets at comparable periods of their reproductive lives.

It can be seen that, on the average, the percentage of pregnancies was not decreased by vitamin B_{12} deficiency below that which obtained on the B_{12}-supplemented rations, nor was it decreased below the percentage on the stock ration. Furthermore, it is evident that vitamin B_{12} deficiency did not increase the percentage of resorptions above that on the B_{12}-supplemented rations, nor significantly above that on the stock ration. There is little evidence, then, to indicate, under these conditions, any noticeable effect of vitamin B_{12} deficiency

[5] We wish to thank Dr. James A. Green, formerly of this laboratory and now of the Department of Anatomy, Medical School, University of North Carolina, for making these examinations.

TABLE 3

Effect of vitamin B_{12} deficiency upon litter size and birth weight of young from mothers on yeast-free rations

RATIONS	EXPT.	NO. OF MOTHERS	SUPPLEMENT	TOTAL NUMBER OF LITTERS	NO. OF LITTERS WHERE YOUNG OBSERVED	AVERAGE NO. OF YOUNG FOUND PER LITTER [1]		AVERAGE BIRTH WEIGHT OF LIVING YOUNG [2]	
						Total	Dead		
								gm	
Stock	..	..	None	..	6977	8.3	0.6	..	
Stock	1–5	161	None	..	251	9.7 ± 0.16 [3]	0.3	5.99 ± 0.05 [3]	(105)
192–195	1	9	None	21	20	7.0 ± 0.62	1.3	5.42 ± 0.11	(13)
192–195	1	4	Liv. ext.	8	8	7.4 ± 0.98	0.2	5.82 ± 0.21	(5)
192–195	2	10	None	19	17	5.4 ± 0.69	1.2	5.66 ± 0.17	(13)
192–195	2	5	B_{12}	10	10	3.4 ± 0.73	0.1	5.81 ± 0.16	(8)
192–195	3	7	None	26	17	5.4 ± 0.85	0.5	5.48 ± 0.24	(10)
192–195	3	7	B_{12}	16	15	4.6 ± 0.59	0.0	5.94 ± 0.26	(7)
192–195	5	5	None	8	8	6.8 ± 0.93	0.1	5.50 ± 0.30	(5)
192–195	5	5	40 × Mn [4]	8	8	6.5 ± 0.91	1.1	5.80 ± 0.16	(6)
192–195	5	8	B_{12}	13	13	6.5 ± 1.02	0.2	6.00 ± 0.25	(7)
193–196	1	8	None	17	16	6.2 ± 0.66	1.1	5.10 ± 0.24	(7)
193–196	1	5	Liv. ext.	9	9	5.9 ± 0.98	0.5	6.12 ± 0.31	(6)
193–196	2	10	None	15	12	5.8 ± 0.87	0.6	5.28 ± 0.19	(5)
193–196	2	5	B_{12}	9	9	3.7 ± 0.65	0.3	5.45 ± 0.27	(4)
300–301	3	5	None	10	10	6.4 ± 0.91	1.0	5.12 ± 0.38	(4)
300–301	3	4	B_{12}	8	8	6.4 ± 0.73	1.2	6.40 ± 0.35	(3)
302–303	3	5	None	10	5	6.6 ± 1.57	0.8	5.13 ± 0.12	(3)
302–303	3	4	B_{12}	8	7	7.9 ± 1.06	0.0	5.56 ± 0.21	(5)
443	5	7	None	14	14	7.3 ± 0.69	0.6	5.68 ± 0.17	(11)
443	5	7	40 × Mn [4]	12	12	5.4 ± 0.77	0.8	5.69 ± 0.24	(8)
443	5	7	B_{12}	11	10	7.7 ± 0.86	1.6	5.86 ± 0.29	(8)
All exptl.	1,2	37	None	..	65	6.2 ± 0.35	1.1	5.42 ± 0.09	(38)
	1,2	19	B_{12} (or liv. ext.)	..	36	5.0 ± 0.48	0.3	5.83 ± 0.12	(23)
All exptl.	3,5	29	None (or 40 × Mn)	..	74	6.2 ± 0.34	0.7	5.55 ± 0.09	(47)
	3,5	30	B_{12}	..	53	6.4 ± 0.41	0.5	5.92 ± 0.12	(30)

[1] Based on the litters where young were actually observed.

[2] The numbers of litters represented are indicated by the figures in parentheses.

[3] Standard error of the mean.

[4] Enough $MnSO_4 \cdot 4H_2O$ added to the ration to bring the total level of added manganese to 40 times that in the other rations.

upon conception or maintenance of pregnancy. No attempt was made in any of these experiments to determine whether vitamin B_{12} deficiency would have altered the length of reproductive life of the animals.

Litter size

The average litter size of the experimental rats, given according to ration and experiment, is shown in table 3 for the yeast-free and in table 4 for the yeast-containing rations;

TABLE 4

Effect of vitamin B_{12} deficiency upon litter size and birth weight of young from mothers on yeast-containing rations

RATIONS	EXPT.	NO. OF MOTHERS	SUPPLEMENT	TOTAL NUMBER OF LITTERS	NO. OF LITTERS WHERE YOUNG OBSERVED	AVERAGE NO. OF YOUNG FOUND PER LITTER [1]		AVERAGE BIRTH WEIGHT OF LIVING YOUNG [2]	
						Total	Dead		
								gm	
194–197	1	9	None	26	24	6.6 ± 0.54 [3]	0.5	5.68 ± 0.13 [3]	(18)
194–197	1	3	Liv. ext.	6	5	3.2 ± 0.74	0.4	6.70 ± 0.10	(2)
194–197	2	9	None	19	19	5.1 ± 0.58	0.1	5.87 ± 0.10	(10)
194–197	2	1	B_{12}	1	1	5.0	0.0	...	...
194–197	3	10	None	23	22	5.4 ± 0.68	0.2	5.72 ± 0.20	(12)
194–197	3	10	B_{12}	24	22	4.9 ± 0.61	0.3	6.39 ± 0.13	(14)
47 [4]	4-Parent	6	None	16	16	8.4 ± 0.90	1.5	5.38 ± 0.56	(5)
45–47	4-F_1	12	None	55	54	6.1 ± 0.40	0.4	5.75 ± 0.15	(35)
45–47	4-F_1	10	Liv. ext.	40	40	7.6 ± 0.43	1.1	5.69 ± 0.11	(28)
45–47	4-F_2	21	None	68	68	7.4 ± 0.29	0.4	5.92 ± 0.08	(45)
45–47	4-F_2	14	Liv. ext.	39	38	8.1 ± 0.50	1.4	5.98 ± 0.10	(17)
45–47	4-F_3	15	None	50	49	5.9 ± 0.33	0.2	5.96 ± 0.11	(31)
45–47	4-F_3	3	Liv. ext.	12	12	7.5 ± 0.89	0.4	6.26 ± 0.38	(5)
445	5	6	None	11	10	8.1 ± 0.66	1.2	5.43 ± 0.22	(3)
All	1,2	18	None	...	43	5.9 ± 0.41	0.3	5.75 ± 0.09	(28)
All	1,2	4	B_{12} (or liv. ext.)	...	6	3.5 ± 0.66	0.3	6.70 ± 0.10	(2)
All	3,4,5	70	None	...	219	6.6 ± 0.19	0.5	5.83 ± 0.06	(131)
All	3,4,5	37	B_{12} (or liv. ext.)	...	112	7.3 ± 0.29	1.0	5.96 ± 0.07	(64)

[1] Based on the litters where young were actually observed.

[2] The numbers of litters represented are indicated by the figures in parentheses.

[3] Standard error of the mean.

[4] In the parent generation of experiment 4, the mothers were maintained on ration 47 at all times and ration 45 was not used.

likewise included in table 3, for comparison, is the record of these same rats before they were placed on the experimental rations and, in addition, an average value for all rats on our stock colony ration over a 5-year period. It can be seen that in every experiment when the mothers were on the purified ration, the average litter size was decidedly less than it was when they were on the stock ration. That this decrease in litter size was greater than would normally be expected is evident from the fact that in our stock colony the average size of the litters cast by a group of several hundred rats decreased from 9.2 for the first litter to 7.5 for the 6th litter; considering that only about 1.5% of the experimental rats were carried beyond the 6th litter, it is noteworthy that in only 7 of the 34 groups did the average litter size equal or exceed this minimum figure. Similarly, comparison with the general stock colony figure of 8.3 (table 3) shows that in only one of the 34 groups did the average litter size of the experimental group equal or exceed this value.

Although there appeared to be a small difference on the average between yeast-free and yeast-containing rations, it is evident that if groups on these rations are compared within each experiment, no consistent difference in litter size can be observed.

If the combined data for the groups on the B_{12}-supplemented rations in experiments 1 and 2 are compared to those for the comparable groups on the B_{12}-deficient rations, a significant decrease in litter size can be observed for both yeast-free ($t = 1.98^{*}$)[6] and yeast-containing ($t = 3.1^{**}$) rations. However, since in these experiments the same animals were placed first on the B_{12}-deficient rations and then on the B_{12}-supplemented rations, some decrease in litter size might be expected if B_{12} supplementation had no curative effect. In experiments 3 and 5 supplementation had no effect on litter size. In experiment 4, however, taking all generations to-

[6] The symbol ** adjacent to a t value indicates statistical significance at or less than the 1% level; * indicates significance at the 5% level or between the 5% and 1% levels; no * indicates no statistically significant difference.

gether, supplementation with liver extract increased the litter size from 6.7 to 7.8 ($t = 3.1^{**}$), the latter figure being somewhat but not greatly below the most nearly comparable stock colony value of 8.3.

The reason for the improved results from supplementation in experiment 4 is not clear. One obvious difference between experiments 4 and 3 is that liver extract was used as a supplement in the former experiment and crystalline vitamin B_{12} in the latter. Although no direct comparisons between liver extract and crystalline B_{12} are available in any of these experiments, comparison of the relative effects of these two supplements on subsequent litter size in experiments 1 and 2 shows that feeding liver extract was associated with an apparent decrease of only 12%, while supplementation with crystalline B_{12} was associated with a decrease of 33%. While the data are hardly sufficient to permit arrival at any final conclusion regarding the comparative effects of these supplements, it is quite evident that the crystalline vitamin produced no improvement at all in the average size of the litters of rats fed these B_{12}-deficient rations.

If all the experiments are combined regardless of ration, no difference in the number of young found dead at birth can be seen between B_{12}-deficient and B_{12}-supplemented groups, the average number being 0.6 for the deficient group and 0.7 for the supplemented group, values which are essentially the same as the general stock colony figure of 0.6.

It can be seen from tables 3 and 4 that there were a number of litters in which no young were observed but which were apparently cast, as judged by a sharp drop in weight at about term, presence of blood on the bedding, etc. About 5% of the total number of litters (34 of 642) fell in this category. Of these 34 litters, 27 were on the B_{12}-deficient rations and 7 were on the B_{12}-supplemented rations. These were almost always (29 of 34) litters which were born during periods when one or two days intervened between observations. The occurrence of such a large proportion of these cases on the B_{12}-deficient rations is undoubtedly related to

the poor survival of the young born to mothers on these rations, as evidenced by the large percentage of deaths that occurred within a few days after birth, a point which will be discussed in a subsequent paper.

Average birth weight

The average birth weights [7] shown in tables 3 and 4 are based upon weighings made from zero to 24 hours after parturition; however, in practically all instances less than 18 hours elapsed before weighing.

While within any one of these experiments no comparisons have been made of the relative effects of liver extract and crystalline B_{12}, comparisons between experiments 1 and 2 indicate no significant difference ($t = 1.3$) in birth weight between the yeast-free groups fed these two supplements. It is likewise evident that similar comparisons between experiments 3 and 4 for the groups fed the yeast-containing rations show no superiority at all for liver extract supplementation over B_{12} supplementation.

If only the yeast-free diets are considered (table 3) in comparing the B_{12}-deficient and the B_{12}-supplemented rations in regard to birth weights, it can be seen that in every group the young from mothers receiving either liver extract or crystalline vitamin B_{12} weighed more than those from the B_{12}-deficient mothers. When all the groups on yeast-free rations are combined, a highly significant difference is observed ($t = 3.6^{**}$),[8] the average being 5.50 gm for the B_{12}-deficient young as compared with 5.88 gm for the B_{12}-supplemented group. The B_{12}-supplemented groups were also significantly higher when either experiments 1 and 2 only ($t = 2.8^{**}$) or experiments 3 and 5 only ($t = 2.4^{*}$) are considered, and it can be seen from table 3 that the average differences between

[7] All the rats in a litter were weighed as a group, the average weight per rat calculated for each litter, and the means of these averages computed to give the figures shown in the tables. The birth weights of all litters which were not weighed within 24 hours after birth have been omitted from the calculations.

[8] See footnote 6, page 386.

B_{12}-supplemented and B_{12}-deficient groups were of about the same magnitude in the two sets of experiments. Thus it is evident that B_{12} prevented the occurrence of these lower birth weights, regardless of whether or not these animals had previously experienced a period of B_{12} deficiency.

With the yeast rations (table 4), comparisons of the birth weights of young from B_{12}-fed mothers with those of young from the corresponding B_{12}-deficient mothers indicate no material difference with some groups, but with others, as was found with the rations containing B vitamins in place of yeast, a higher average value for the B_{12}-fed mothers. The groups in which these higher averages occurred were those run at a later period — experiment 4, F_3 generation, and experiments 1 and 3. This difference was significant ($t = 2.8^{**}$)[9] only in experiment 3. It will be noted that the different results in the various experiments are attributable primarily to higher values for the B_{12}-fed groups in the later experiments, rather than to a change in the values for the B_{12}-deficient groups. Some of the apparent inconsistency of these results may have been due to variability, in some respect, of the different purchase lots of yeast used in the various experiments.

If the results for all the groups on the yeast-containing rations are combined, the average birth weight for the B_{12}-deficient young is 5.82 gm as compared with 5.99 gm for the B_{12}-supplemented young, a difference in favor of the B_{12}-supplemented young but one that is not quite statistically significant ($t = 1.9$). It can be seen that the birth weights on the yeast-containing B_{12}-supplemented rations were on the average as high as those obtained on the stock ration.

It will also be noted that the average birth weight of the young from mothers on the yeast-containing rations was higher than that of the young from mothers on the yeast-free rations, whether B_{12}-deficient groups or B_{12}-supplemented groups are compared. This difference is highly significant

[9] See footnote 6, page 386.

($t = 3.9^{**}$)[10] when B_{12}-deficient rations are considered, but much smaller and not significant ($t = 0.9$) when B_{12}-supplemented rations are compared. If the comparisons are confined to experiment 3, however, the average difference between yeast-free and yeast-containing rations is of about the same magnitude and just as significant whether B_{12}-deficient or B_{12}-supplemented diets are considered. It would seem improbable that these different results could be attributed to the presence of vitamin B_{12}-active material in some lots of yeast. The rat growth assay method used in this laboratory has given little if any indication of such activity at any time. Microbiological methods (Peeler et al., '51) have likewise shown almost complete absence of vitamin B_{12} from dried brewers' yeast.

SUMMARY

Results have been presented dealing with certain aspects of reproduction in rats fed vitamin B_{12}-deficient casein-containing rations supplemented with all other known nutrients. With females on these rations there were no greater percentages of non-pregnancies or resorptions than there were with females on the stock ration, but the average size of their litters was smaller and their young generally had lower average birth weights. Supplementation of the B_{12}-deficient rations with crystalline vitamin B_{12} had little or no effect on litter size but did increase birth weights. Birth weights were on the average higher on yeast-containing rations than on yeast-free rations.

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[10] See footnote 6, page 386.

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EFFECT OF FRYING BACON ON THE NUTRITIVE VALUE OF THE PROTEIN[1]

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The importance of evaluating the effect of processing on the nutritive value of foods has been well recognized. Analytical studies and animal experiments have been directed toward determination of the nutrient content as well as the completeness of utilization of specific nutrients by the animal. The present study was undertaken to determine the effect of frying bacon on the nutritive value of the protein of bacon, based on the rate of growth and efficiency of utilization of the protein of uncooked and cooked bacon by the rat.

EXPERIMENTAL

Sliced bacon, in one-half-pound packages, was purchased on the open market. The bacon was obtained from two sources and each lot was divided into two equal portions; one portion was cooked and the other was retained as the uncooked control sample. The bacon to be cooked was pan-broiled at high heat until considered crisp or well-done (approximately 5 min. cooking time), cooled and ground. The uncooked bacon

[1] Journal Paper 40, American Meat Institute Foundation. The proximate analyses were made by the Service Laboratory, American Meat Institute Foundation. We are indebted to Merck and Co., Rahway, New Jersey, for the vitamin B_{12}, to Lederle Laboratories, American Cyanamid Co., for the pteroylglutamic acid, and to E. I. DuPont de Nemours and Co., Inc., for the DL-methionine used in these studies.

was ground, lyophilized, and then both the uncooked and cooked bacon were extracted with ether in the cold. The samples were then dried, ground and analyzed for crude protein (N × 6.25).

Proximate analyses were made on samples of the uncooked and cooked products prior to drying and ether extraction.

The dried, ether-extracted products were then incorporated into rations to provide a 10% level of crude protein as the sole source of protein in the ration. These rations were then

TABLE 1

Composition of basal ration

INGREDIENTS	%	INGREDIENTS	*mg/100 gm ration*
Salts IV [1]	4	Thiamine HCl	0.6
Corn oil	4.7	Riboflavin	0.6
Fish liver oil (3,000 A, 400 D/gm)	0.3	Pyridoxine HCl	0.6
		Ca pantothenate	4.0
		Nicotinic acid	2.0
Dried, ether-extracted bacon } to	100	Choline chloride	100.0
Sucrose } to		Inositol	100.0
		Pteroylglutamic acid	0.2
		Biotin	0.01
		2-methyl 1,4 naphthoquinone	3.0
		p-Aminobenzoic acid	30.0
		Vitamin B_{12}	0.002

[1] Hegsted et al. ('41).

fed to weanling male rats of the Holtzman strain for a three-week period. Seven rats were used in each group, food and water were provided ad libitum, and the rates of gain and food efficiency for each group determined. Lactalbumin to provide 10% protein in the ration was fed to a control group of animals. The composition of the basal ration used in these studies is given in table 1.

A second experiment was conducted with two additional lots of bacon. In this study the bacon was oven-broiled in order

to provide more controlled conditions of cooking, and an additional test was conducted with one lot of bacon in which crisp frying (well-done) was compared to limp frying (medium-done). For the crisp-fried samples, the bacon was broiled in electric ovens equipped with Veriac transformers to provide precise control of current to the broiling units. The temperature at the surface of the broiling pan was maintained at 177°C. The cooking time for the crisp-fried samples

TABLE 2

Proximate analysis of cooked and uncooked bacon

(all values in per cent)

SAMPLE	PROTEIN	MOISTURE	ETHER EXTRACT	ASH
Experiment 1				
Bacon A (uncooked)	6.72	17.4	74.0	1.3
Bacon A (pan-broiled)	30.50	5.4	54.8	6.8
Bacon B (uncooked)	8.40	19.2	70.0	2.0
Bacon B (pan-broiled)	30.73	5.4	54.7	8.6
Experiment 2				
Bacon C (uncooked)	7.18	17.8	73.2	2.1
Bacon C (oven-broiled, crisp)	23.93	9.4	58.9	6.7
Bacon C (oven-broiled, limp)	18.99	13.6	61.3	5.5
Bacon D (uncooked)	8.06	19.1	69.1	2.0
Bacon D (oven-broiled, crisp)	31.03	10.2	49.4	7.1

was 5½ min., and for the limp-fried samples, 4 min. The weight loss of the bacon during cooking was 71 and 74% for the two lots that were crisp-fried and 62% for the lot that was limp-fried.

The preparation of the samples, composition of the basal ration, and regimen for the feeding experiment were the same as those used in the first experiment. The experiment was conducted for a period of 24 days. The rate of growth and food efficiency were determined.

The proximate analyses for the uncooked and cooked samples are presented in table 2. A summary of the feeding tests

on the effect of cooking on the utilization of the protein of bacon is presented in table 3 for the first experiment and table 4 for the second experiment.

TABLE 3

Effect of frying of bacon on the growth rate and food efficiency of rats

(Experiment 1)

TEST PRODUCT	AVE. GAIN/WEEK [1]	FOOD EFFICIENCY
	gm	*gm gain per gm food consumed*
Bacon A (uncooked)	23.3	0.29
Bacon A (cooked)	19.3	0.25
Bacon B (uncooked)	21.2	0.27
Bacon B (cooked)	20.1	0.25
Lactalbumin	30.0	0.36

[1] Seven rats per group, 21-day experimental period (see text).

TABLE 4

Effect of crisp- or limp-frying of bacon on the growth rate and food efficiency of rats

(Experiment 2)

TEST PRODUCT	AVE. GAIN/WEEK [1]	FOOD EFFICIENCY
	gm	*gm gain per gm food consumed*
Bacon C (uncooked)	19.4	0.32
Bacon C (crisp-fried)	18.7	0.29
Bacon C (limp-fried)	19.7	0.33
Bacon D (uncooked)	19.8	0.32
Bacon D (crisp-fried)	19.8	0.31

[1] Seven rats per group, 24-day experimental period.

RESULTS AND DISCUSSION

The data presented in tables 3 and 4 show that the rats grew at a rate approximating 19 to 23 gm per week when fed either cooked or uncooked bacon. The reference group fed lactalbumin as the sole source of protein grew at a rate of 30 gm per week, with a food efficiency of 0.36. In all cases the test product was added to provide 10% of crude protein

in the diet. That this level of protein was the limiting factor for growth was indicated by the lactalbumin results, and permitted detection of changes in the nutritive value of the protein of the bacon that may be attributed to sample treatment.

The results obtained with respect to the rates of gain and food efficiency were analyzed statistically by the analysis of variance method. There was no reduction in the nutritive value of the protein of the bacon attributable to cooking for bacon sample B of the first experiment, bacon C (crisp- or limp-fried) or bacon D (crisp-fried) used in the second experiment, evaluated on the basis of the rate of gain of the animals, or food efficiency. The reduction in protein quality for bacon A of the first experiment was statistically significant both on the basis of rate of gain and food efficiency, as compared to the uncooked sample. The F values observed for the effect of cooking bacon A were 6.22 (rate of gain) and 9.75 (food efficiency), while the F value required at the 5% level of significance was 4.67 and at 1% was 9.07. Thus it may be concluded that the crisp frying of bacon under the conditions outlined reduced the nutritive value of the protein slightly for one of the 4 samples, and that when the data were evaluated by statistical analysis, the reduction was more significant when based on food efficiency data. The latter circumstance undoubtedly is a reflection of reducing the variation of response within the groups, since the animals that gained the most rapidly also consumed the most food and vice versa, and this resulted in less variation in the food efficiency data within each experimental group.

It is recognized that determinations of the rate of gain, food efficiency or protein efficiency with a given protein source are a reflection only of the amino acid, or acids, that is present or available to the animal in the most limiting amount. The utilization of other amino acids that are not limiting may be either decreased or increased by a specific treatment of the samples, and would not be detected by this method. On the basis of comparisons of amino acid requirements and

the amino acid composition of muscle proteins (Mitchell and Block, '46) and, more specifically, the amino acid content of bacon protein (Schweigert, Bennett, McBride and Guthneck, '50), it appeared likely that the sulfur-containing amino acids might be limiting the rate of growth. Therefore an additional group of rats were fed bacon C, crisp-fried, plus 0.3% DL-methionine in the second experiment, in addition to the basal ration. This group gained an average of 21.3 gm per week, with a food efficiency of 0.39, as compared to 18.7 and 0.29, respectively, for the group receiving the crisp-fried bacon without methionine supplementation. These findings suggest that the sulfur amino acids were limiting the rate of growth, but that other amino acids then became limiting, which permitted only a small response to added methionine.

SUMMARY

The effect on the nutritive value of the protein of bacon attributable to cooking was investigated. Lyophilized and ether-extracted bacon was fed as the sole source of protein at a 10% level in the diet to weanling rats. No reduction in the utilization of the protein by weanling rats was observed in three tests with crisp-fried (well-done) bacon or in one test with limp-fried (medium-done) bacon as compared to control groups receiving uncooked bacon. A small reduction in the nutritive value of the protein, which was statistically significant, was observed for one test with crisp-fried bacon.

The per cent weight losses of the bacon during cooking and the proximate analyses of the cooked and uncooked products are presented.

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THE UTILIZATION OF DIETARY CALCIUM BY GROWING ALBINO RATS FED DIETS CONTAINING LARD OR COCOA BUTTER[1]

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In a paper by Steggerda and Mitchell ('51), a study of the effect of dietary levels of milk fat ranging from 1 to 32% (dry basis) on the calcium retention of adult human subjects was reported. Although 13 subjects were included in the study, and the response of each subject to both high- and low-fat diets was secured in experimental periods generally of 16 days' duration, no effect of fat on calcium metabolism was demonstrated under the conditions imposed. In the present paper will be reported the effect of the kind of dietary fat on calcium utilization, using the growing albino rat as subject. Two fats differing rather widely in physical and chemical properties, lard and cocoa butter, were selected for this purpose.

From available information it would be expected: (1) that if any effect were exerted, it would depend to a large extent upon the formation of insoluble calcium soaps; and (2) that the formation of such soaps would be most likely to occur with fats that are relatively indigestible. Using a modified Cori method and the rat as subject, Steenbock, Irwin and Weber ('36) demonstrated marked differences in absorption among a wide array of fats. After a 4-hour absorption period the mean percentages of fat absorbed, with their probable

[1] This investigation was aided by funds donated to the University of Illinois by the National Dairy Council in behalf of the chocolate ingredient manufacturers.

[2] Deceased.

errors, ranged from 67.0 ± 0.9 for raw linseed oil to 35.8 ± 1.0 for oleo stock. For the two fats used in the present study, the corresponding values were 57.0 ± 1.5 for lard and 47.9 ± 1.1 for cocoa butter. Much other evidence could be cited to the same effect.

Calverley and Kennedy ('49) have demonstrated different effects of various fats on calcium retention in growing rats. Expressed as percentage retentions of calcium on intake, the average values, with standard deviations, secured with 12 rats to each diet in 28 days of feeding were: 63 ± 1.7 for peanut oil, 59 ± 2.7 for coconut oil, and 55 ± 2.6 for completely hydrogenated cottonseed oil. All of these values are lower than that secured with a no-fat diet, i.e., 65 ± 4.3. The mean differences between the diets containing 5% of hydrogenated cottonseed oil or 5% of coconut oil and the no-fat diet were highly significant statistically, but that between the peanut oil diet (5% fat) and the no-fat diet was not significant. Calcium soaps of dietary origin were present in considerable amounts in the feces from the hydrogenated cottonseed oil diet, in slight amounts in the feces from the coconut oil diet, and were absent from the feces from the peanut oil diet. Using mature rats, Westerlund ('34a, b) has demonstrated an effect of tripalmitin, but not of triolein or tributyrin, on the output of fecal calcium.

The experiment to be reported below is one of a series concerned with an attempt to explain how a certain low-cost, low-fat cocoa impaired calcium utilization in growing rats "to an extent three times as great as could be accounted for by its content of oxalic acid" (Mitchell and Hamilton, '46). It was shown that not all low-fat cocoas exerted this effect. In fact, two cocoas [3] prepared from the same cacao beans but containing 12.55% ether extract in one case and 23.16% in the other case, exerted practically identical depressive ef-

[3] These cocoas, as well as the cocoa butter used in the experiments reported, were prepared especially for this investigation by the Peter Cailler Kohler Swiss Chocolate Co., Inc., of Fulton, N. Y., through the courtesy of Dr. R. F. Korphage.

fects on calcium rentention when incorporated into the experimental diets to the same extent, namely, 16%. In another experiment, theobromine incorporated in the experimental diet to the extent of 0.32%, equivalent to the content of this alkaloid in 16% of cocoa, was not found to exert a significantly deleterious effect on calcium retention, which averaged 66.9 for the control rats and 64.4 for the theobromine rats. The next step was to compare the effect on calcium utilization of cocoa butter and some other fat commonly used in the American diet, i.e., lard. This is the subject of the present paper.

It will be shown that 20% of lard in the diet does not appreciably depress the utilization of dietary calcium below that observed on a low-fat (1%) diet, while 20% of cocoa butter depresses calcium utilization by some 22%.

EXPERIMENTAL METHODS

Twelve trios of male albino rats were fed for 42 days in a comparison of three diets, one containing only 1% fat, the second an additional 20% of lard, and the third an additional 20% of cocoa butter. The test fats were incorporated into the diets at the expense of starch and glucose. The diets contained 0.312 to 0.351% calcium and were otherwise complete nutritionally. The physical and chemical compositions of these diets are given in table 1.

The three rats in each trio were fed the same amount of their respective diets throughout the 42-day feeding period. In order to equalize the energy intakes of the trio the rat on the low-fat basal diet was given added sucrose or dextrin [4] in amounts to maintain the same rate of growth exhibited by his trio mates. In fact, the gains in body weight of all three trio mates were equalized as far as possible by giving appropriate doses of carbohydrate in order to minimize the effect of variable growth rate on calcium utilization (Fairbanks and Mitchell, '36).

[4] Amidex.

One rat from each of the litters supplying the experimental rats was sacrificed at the beginning of the experiment and analyzed for calcium to permit estimates of the initial calcium content of the experimental animals. The latter were then killed with ether at the termination of the feeding period and analyzed for calcium. The calcium retained during the feeding period was then computed in grams and in per cent of calcium consumed.

TABLE 1

Composition of experimental diets

COMPONENTS	DIET		
	Low-fat	Lard	Cocoa Butter
	%	%	%
Dried extracted whole egg	15	15	15
Non-fat milk solids	20	20	20
Salt mixture, Ca- and P-free	2	2	2
Sodium chloride	1	1	1
Wood flock	2	2	2
Vitaminized starch	5	5	5
Starch, corn	22	12	12
Cerelose (glucose)	22	12	12
Wheat germ oil	0.5	0.5	0.5
Cod liver oil	0.5	0.5	0.5
Lard	..	20	..
Cocoa butter	..	..	20
Sucrose	10	10	10
Total	100	100	100
Chemical composition			
Protein (N × 6.25)	18.94	19.38	21.56
Calcium	0.312	0.318	0.351
Gross energy, cal. per gm	3.91	5.04	5.17

During one week of the experiment the feces of all rats were collected for analysis for moisture, nitrogen and calcium and for determination of the heat of combustion in the bomb calorimeter. The calcium in diets, carcasses and feces and the nitrogen in feces were determined by slight modifications of the methods of the Association of Official Agricultural Chemists.

DISCUSSION OF EXPERIMENTAL RESULTS

The average experimental results pertaining to the growth, food consumption and calcium utilization of the rats are assembled in table 2. The rats grew at a rate of 3.7 to 3.8 gm daily. The attained body length from nose to root of tail averaged the same on all diets, i.e., 202 mm. However, these results were secured with a smaller average caloric intake of the lard diet, i.e., 1798 cal., than on either the low-fat diet, 1861 cal., or the cocoa butter diet, 1861 cal. Comparisons within

TABLE 2

The average growth, food consumption and calcium utilization of the experimental rats

	GROUP 1 BASAL LOW-FAT DIET	GROUP 2 20% LARD DIET	GROUP 3 20% COCOA BUTTER DIET
Number of rats	12	11[1]	12
Days on test	42	42	42
Initial body weight, gm	46	46	46
Final empty body weight, gm	202	204	202
Average daily gain, gm	3.7	3.8	3.7
Total food consumption, gm			
Experimental diet	354	353	354
Sucrose	93	6	5
Amidex (dextrin)	27	0.2	3.4
Energy, cal.	1,861	1,798	1,861
Total calcium intake, mg	1,108	1,121	1,242
Total calcium retained, mg	816	806	717
Total calcium retained, %	73.9	72.1	57.6

[1] One rat in trio 10, receiving the lard diet, died early in the experiment.

trios showed that the average difference between the low-fat and the lard diets was significant ($t = 2.02$, $P = 0.036$) and that that between the lard and the cocoa butter diets was highly significant ($t = 2.85$, $P = 0.009$). These and other probabilities given below were taken from Student ('25).

The average calcium utilization, expressed as percentage of the intake that was retained, was 73.9 for the low-fat diet, 72.1 for the 20% lard diet, and 57.6 for the cocoa butter diet. Again taking diet differences within trios, the mean differ-

ence between the low-fat and the lard diets was statistically insignificant ($t = 0.66$, $P = 0.26$), while the mean difference between the low-fat and the cocoa butter diets ($t = 4.52$, $P = 0.0005$), as well as that between the lard and the cocoa butter diets ($t = 3.67$, $P = 0.0024$), was highly significant.

The impairment in calcium utilization brought about by 20% of cocoa butter in the diet, while a similar level of dietary lard occasioned no appreciable effect, is not associated with a higher melting point of the former fat. Cocoa (or cacao) butter has a melting point of 32 to 34°C. (American Meat Institute, '50). A lower digestibility of cocoa butter than of lard would not be expected. Nevertheless, the dry weight of feces was higher in all trios for the cocoa butter rat than for the lard rat, 20.40 compared with 14.14 gm per week, or for the low-fat rat, 13.50 gm per week. The heat of combustion of the dry feces averaged 4.73 cal. per gram for the low-fat diet, 4.87 cal. per gram for the lard diet, and 5.81 cal. per gram for the cocoa butter diet; the differences among all of these means are significant statistically, since in all pairs but one there was no non-conformance, the one exception relating to a low-fat lard comparison. The calcium in the feces averaged 10.5, 16.0 and 33.7% of the intake, for the low-fat, lard, and cocoa butter diets, respectively, and the diet differences were highly significant. Hence, although neither fecal fat nor fecal calcium soaps were determined directly, it may be fairly assumed that the deleterious effect of cocoa butter upon calcium utilization is due to the formation of insoluble calcium soaps in the intestinal tract.

SUMMARY AND CONCLUSIONS

The comparative effect on calcium utilization of lard and of cocoa butter administered at a 20% dietary level, and of these diets as compared with a low-fat diet, was studied using 12 trios of young albino rats in a 42-day feeding period. The effect was measured as total retention of calcium expressed as a percentage of the intake of this element. The

experimental diets were fed in equal amounts to trio mates and the gains in body weight were equalized by supplements of sucrose or dextrin administered as needed.

The average calcium utilization was 73.9 for the low-fat diet, 72.1 for the lard diet and 57.6 for the cocoa butter diet. The difference between the first two values was insignificant statistically, while the differences between the first and the third values, and between the second and third values, were highly significant. These differences relate, from the standpoint of human nutrition, to inordinate levels of cocoa butter in the diet.

From measurements secured of the dry weight, calcium content, and heat of combustion of the feces produced on these three diets, it is evident that the inhibiting effect of cocoa butter on calcium utilization is largely, or entirely, accounted for by the greater formation of insoluble calcium soaps.

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THE EFFECT OF LEVEL OF INTAKE ON CALCIUM AND PHOSPHORUS METABOLISM IN COLLEGE WOMEN [1, 2]

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A major nutrition problem today is that of supplying the amounts of calcium which authorities recommend for good nutrition. Studies on calcium metabolism, carried out for the most part on lower animals, have shown that many factors and conditions influence calcium absorption, retention, and urinary excretion. Since this mineral is not readily soluble, any factor or condition which increases its solubility, such as increased intestinal acidity, tends to improve calcium absorption; and any factor which decreases its solubility, such as the presence of oxalates, phytates, or phosphates, tends to reduce intestinal absorption. With increase in absorption, more of this element is made available to the body, but its retention by the tissues is dependent on the presence of adequate amounts of those ions, elements, and compounds which are needed for its utilization. Metabolized calcium and absorbed calcium which for some reason is not retained by the tissues are eliminated by the kidneys. The gastrointestinal tract may also serve as an excretory organ for this element.

[1] Paper 2295, Scientific Journal Series. Minnesota Agricultural Experiment Station.

[2] Journal Article J-24-51, Ohio Agricultural Experiment Station.

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Excretion of calcium in the feces in excess of the dietary intake was observed by Steggerda and Mitchell ('46a, b), who considered this suggestive evidence that all of the fecal calcium is not unabsorbed dietary calcium. Stearns ('50) pointed out that the calcium content of the digestive secretions varies from 5 to 10 mg per 100 ml, and estimated that from 0.3 to 0.8 gm, with a mean of about 0.65 gm, of calcium is secreted into the gastrointestinal tract daily. She stated that if conditions in the tract do not favor absorption, much of the calcium of the digestive secretions, as well as that of the food, will be excreted and, in consequence, fecal excretion may greatly exceed the intake.

Man's low efficiency in the utilization of dietary calcium as compared with the lower animals presents a problem for which there is at present no adequate explanation. The present investigation was undertaken for the purpose of helping to clarify this problem.

PROCEDURE

The subjects for the study were healthy college women ranging in age from 20 to 30 years. Eight were students at The Ohio State University and 9 at the University of Minnesota. Each student was given a physical examination by the University Student Health Service before being accepted as a subject, to insure that only healthy individuals were included. No student receiving any kind of medication (iron, iodine, vitamins, etc.) was included. The study was carried out during the winter of 1948.

The basal diet, which yielded about 2,140 cal. and 50 gm protein, was planned to supply approximately 300 mg of calcium and 900 mg of phosphorus per day. These levels were chosen as representing the lowest levels compatible with the serving of appetizing meals. The diet was planned on a 7-day basis and was repeated for each of the 8 balance periods. A typical day's menu is given in table 1. Sugar was allowed ad libitum, but a record was kept of the amount consumed by each subject.

Distilled water was used for food preparation and drinking purposes and chemically pure sodium chloride as seasoning. The subjects were requested to use this salt as a dentifrice, since many tooth pastes and powders contain appreciable amounts of calcium.

The calcium content of the basal diet was found on analysis to be in good agreement with the calculated value. The phosphorus content, however, was somewhat less than planned

TABLE 1

A typical day's menu

BREAKFAST		LUNCHEON		DINNER	
Food	Amount	Food	Amount	Food	Amount
	gm		*gm*		*gm*
Grapefruit, canned	100	Grapejuice	100	Leg of lamb	85
Toast, white bread	25	Baked egg with	50	Potato, boiled	100
Butter	7½	ham and	60	Gravy	50
Cereal, prepared	15	cream	15	Peas, frozen	100
Cream, light	60	Salad, molded, tomato	100	Salad, lettuce	40
Jam	20	lettuce	10	French dressing	11
Coffee		mayonnaise	14	White bread	25
		White bread	25	Butter	15
		Cracked wheat bread	25	Apple pudding	100
		Butter	15	Cream, light	35
		Pear, canned	100	Tea	
		Tea			

and averaged slightly more than 800 mg daily. In consequence, the calcium to phosphorus ratio in the basal diet was 0.38 instead of 0.33, as planned.

Small variations in calcium and phosphorus intake occurred among the subjects since foods low in these minerals were allowed ad libitum to those individuals whose energy requirement exceeded the caloric content of the basal diet.

During the first 4 weeks of the study all subjects received the same basal diet. During the next 4 weeks, 5 subjects (two at Ohio and three at Minnesota) served as controls (group I) and were continued on the same diet; 6 subjects, three at each location (group II), were given daily a mineral supplement

which supplied 1,200 mg of calcium (treatment 1) [4]; and the remaining 6 subjects, three at each location (group III), received mineral supplements which supplied 1,200 mg of calcium and 600 mg of phosphorus (treatment 2) [5]. Due to the fact that the phosphorus content of the basal diet was lower than planned, the calcium to phosphorus ratios were 1.85 and 1.06 instead of 1.67 and 1.00 for treatment periods 1 and 2, respectively. The subjects received daily a 1-mg tablet of riboflavin and 400 I.U. of vitamin D.[6]

Servings of each of the foods were weighed on a torsion balance to the nearest tenth of a gram. Food composites for analysis were prepared at the time the foods were served to the subjects.

Total urine and fecal collections were made. Acid digests were prepared of the food and fecal composites, using the procedure of Stearns ('29).

Calcium determinations were made using a procedure based on the Stearn's ('29) modification of the McCrudden method. For phosphorus, the Fiske and Subbarow ('25) molybdate method was used. The color was read in a photoelectric colorimeter and phosphorus content was computed from a constant which had been determined from standard phosphorus solutions.

Insofar as possible, identical procedures were followed in all aspects of the study by the Ohio and Minnesota workers. Food and fecal digests were exchanged and analyzed. The results showed no significant differences in the values obtained by the two laboratories.

[4] This supplement consisted of 4 calcium carbonate tablets, each containing 300 mg of calcium. One tablet was given with breakfast, one with lunch, and two with dinner.

[5] This supplement consisted of two calcium carbonate tablets and 4 tablets of dibasic calcium phosphate and dibasic sodium phosphate. One-half of a calcium carbonate tablet and one of the phosphate tablets were given with breakfast and also with lunch; the remainder were taken with dinner.

[6] The authors acknowledge their indebtedness to Mead, Johnson and Company for the vitamin D used in this study.

RESULTS AND DISCUSSION

Calcium metabolism on a constant low intake

Although adjustment periods of three to 5 days have frequently been employed in balance studies of this type, earlier observations in our laboratories had indicated that because of the difficulty in separating precisely the carmine-marked fecal specimens, such short observation periods introduce a considerable error into the data, and this error is particularly serious when the number of subjects used must be limited. A 7-day period was found to reduce this error considerably and therefore was employed in the present study.

The means for each diet period for the subjects from the two states are presented in table 2. The data for the first 4 weeks were used as a basis for establishing the time required for adjustment to the calcium level of the basal diet. Analysis of variance applied to the weekly data from the individual subjects was used to test the significance of the observed differences. The results showed that fecal excretion of calcium in week 1 was significantly greater than in weeks 2, 3, and 4, but there was no difference between the two states.

When the calcium retention data for the subjects in the two states were tested, however, it was found that the Ohio women retained significantly less calcium than did the Minnesota women. That the means for the two groups actually did not differ greatly is evident from the weekly retentions, in milligrams given below:

Weeks	1	2	3	4
Ohio	— 212	— 66	— 58	— 81
Minnesota	— 142	— 19	— 36	— 46

Since the states differed significantly, variances attributable to weeks were studied separately using the Ohio and Minnesota retention data. These tests showed that, at each location, calcium retention in the first observation period differed significantly from that in the second period, but no differences were demonstrated between succeeding periods. From these

findings it is evident that one week is an adequate period for adjustment to a calcium intake as low as that employed in the basal diet.

The calcium retention data for weeks 2, 3, and 4 were also tested for differences between subjects. These showed highly significant differences between the subjects within the two

TABLE 2

Effect of calcium and phosphorus intake levels on calcium metabolism

DIET	WEEK	INTAKE	EXCRETION		BALANCE
			Fecal	Urinary	
		mg	*mg*	*mg*	*mg*
		Group I (5 subjects)			
Basal	1	281	400	136	— 255
	2	307	216	135	— 44
	3	311	221	143	— 54
	4	293	212	149	— 68
Basal	5	311	228	150	— 67
	6	315	199	141	— 24
	7	312	247	137	— 73
	8	329	335	144	— 150
		Group II (6 subjects)			
Basal	1	282	306	125	— 150
	2	311	221	126	— 36
	3	306	236	126	— 56
	4	290	219	121	— 50
Basal + 1.2 gm Ca	5	1,506	992	260	254
	6	1,518	1,020	226	271
	7	1,505	1,098	213	194
	8	1,530	1,207	215	107
		Group III (6 subjects)			
Basal	1	282	317	103	— 138
	2	310	241	113	— 45
	3	306	218	117	— 30
	4	288	243	116	— 71
Basal + 1.2 gm Ca and 0.6 gm P	5	1,506	1,142	176	188
	6	1,517	1,282	165	70
	7	1,506	1,312	144	50
	8	1,532	1,414	138	— 20

states. Because of this wide individual variation in calcium utilization by human subjects, investigators have customarily employed groups of subjects as large as could be managed. The number of observation periods has also varied greatly in different investigations, some extending over many months, making such studies not only very arduous but also very expensive. To answer the question as to whether it might be better in another experiment of this type to have more subjects for fewer periods, or to have fewer subjects for more periods, the data on retention from both Ohio and Minnesota were so analyzed that variances due to subjects and to weeks were separated from error variance. The data for the first week were not included in these analyses. The variance among subjects for the Ohio group was not significantly greater than error variance. For the Minnesota group the variance among subjects, although smaller than that for the Ohio group, was significantly greater than error variance. In both states the variance due to weeks did not differ significantly from error variance. These findings suggest that in another experiment it would be possible to reduce the number of periods, but that it would be unwise to decrease the number of subjects.

Phosphorus metabolism on a constant low intake

Table 3 gives the values for phosphorus which are comparable to those for calcium in table 2. By means of variance analysis it was shown that fecal excretion of phosphorus in the first week was significantly greater than in the succeeding three weeks, but no difference in response was demonstrated between states or between subjects within states.

Phosphorus retention, like that of calcium, was also significantly different in week 1 from that in weeks 2, 3, and 4. No difference between weeks or between subjects within the two groups was found when the data from the first week were excluded. One week was, therefore, an adequate period for the adjustment of phosphorus metabolism to the level of intake employed in the basal diet of this study. The marked

difference in phosphorus retention between week 1 and weeks 2, 3, and 4 is evident from the following tabulation of the mean daily retentions in milligrams per subject:

Weeks	1	2	3	4
Ohio	— 136	10	37	30
Minnesota	— 112	4	4	— 17

The differences between the retention values for the Ohio subjects and the Minnesota subjects during the last three

TABLE 3

Effect of calcium and phosphorus intake levels on phosphorus metabolism

DIET	WEEK	INTAKE	EXCRETION		BALANCE
			Fecal	Urinary	
		mg	*mg*	*mg*	*mg*
		Group I (5 subjects)			
Basal	1	747	388	538	— 178
	2	790	273	509	9
	3	820	270	536	15
	4	795	257	538	—
Basal	5	823	292	518	13
	6	830	248	534	48
	7	812	304	585	— 77
	8	823	366	556	— 98
		Group II (6 subjects)			
Basal	1	740	300	520	— 80
	2	792	249	527	16
	3	810	263	525	23
	4	788	244	538	7
Basal + 1.2 gm Ca	5	809	352	392	65
	6	813	389	390	34
	7	799	368	410	21
	8	813	391	426	— 4
		Group III (6 subjects)			
Basal	1	740	311	537	— 107
	2	792	273	522	3
	3	811	261	529	21
	4	788	265	538	— 16
Basal + 1.2 gm Ca and 0.6 gm P	5	1,410	612	738	60
	6	1,414	670	740	4
	7	1,400	634	712	54
	8	1,417	680	754	— 18

weeks were significant at the 5% level. It is interesting to note that whereas negative calcium balances were observed in weeks 2, 3, and 4, positive phosphorus balances were recorded except for the Minnesota group in the 4th week.

Urinary excretion of calcium and phosphorus on a constant low intake

It is generally assumed that calcium absorption is the factor of primary importance in determining calcium balance. The data secured in the present study indicate that urinary calcium excretion is also of considerable importance in determining calcium balance. It is recognized that urinary calcium may be metabolized calcium or it may represent absorbed calcium which is not retained due to the lack of one or more of the factors needed for its storage or use, or because the amount absorbed exceeds the body's need.

The urinary calcium excretion levels of the subjects in this study varied widely and appeared to be characteristic of the individual. This can be most readily illustrated by the data for the 5 subjects who were maintained on the low calcium intake for the entire 8 weeks of the study. One subject (M-2) had an unusually low urinary calcium excretion level, the mean daily loss during weeks 2, 3, and 4 and 6, 7, and 8 amounting to 18 mg; the range was from 15 to 21 mg. Subject O-2 had the highest urinary excretion level of this group, the corresponding mean being 232 mg and the range being from 212 to 242 mg. It is evident that, although these subjects were on the same diet, subject O-2 lost more than 10 times as much calcium in the urine as did subject M-2. This difference in urinary excretion was associated with a corresponding difference in calcium balance, the former subject (O-2) showing a mean daily balance for the 6 weeks of — 90 mg and the latter (M-2) a mean daily balance of — 15 mg.

The subjects in this study showed very much less variation in urinary phosphorus excretion than in calcium excretion. Subject O-2, for example, had a mean daily urinary phos-

phorus excretion for the 6 weeks of 505 mg, the lowest in the group, and subject M-2 of 513 mg. The highest mean value noted for any subject was 590 mg.

Effect of increased intake on calcium and phosphorus metabolism

Increasing the calcium intake from approximately 300 mg daily to 1,500 mg without altering the phosphorus intake (treatment 1) resulted in a marked increase in calcium retention; i.e., from a mean of — 47 mg in weeks 2, 3, and 4 to a mean of 191 mg in weeks 6, 7, and 8, an increase of 238 mg. Despite the higher calcium retention during the weeks of calcium supplementation, urinary excretion of calcium was also increased. Phosphorus retention, on the other hand, was not altered, but the urinary excretion of this element was reduced.

When both calcium and phosphorus were added to the basal diet (treatment 2), the change in calcium retention was from a mean of — 49 mg in weeks 2, 3, and 4 to a mean of 33 mg in weeks 6, 7, and 8, an increase of 82 mg. Calcium excretion in the urine was also increased. It is evident that increasing the phosphorus intake from 800 mg to 1,400 mg resulted in much less satisfactory utilization of calcium in the subjects on treatment 2 than in those on treatment 1. Since this amount of phosphorus is little more than the daily allowance customarily recommended for adults, it would appear that a factor of considerable importance in the relatively poor utilization of calcium by human subjects is the amount of phosphorus regularly included in the diet.

The increased phosphorus intake on treatment 2 had little effect on phosphorus retention, the mean values for weeks 2, 3, and 4 and for weeks 6, 7, and 8 being 1 mg and 14 mg, respectively. Urinary excretion of phosphorus was greatly increased, however. These findings indicate that, for the subjects in this study, 800 mg comprised an adequate daily intake of this element.

Since, at least in the Minnesota subjects, there was evidence, as pointed out earlier, that the group was not homogeneous, it was necessary to have each subject serve as her own control in order to determine whether or not the observed effects of the calcium and phosphorus supplements on the retention of these minerals were statistically significant. For each subject the mean daily calcium retention on the basal diet was subtracted from the retention during the corresponding week when the supplement was given. The differences were assumed to be due to the treatment effect. The phosphorus data were treated in the same manner. A two-way analysis of variance applied to these data showed that significantly more calcium was retained by the subjects on treatment 1 than by those on treatment 2, but there was no difference in phosphorus retention. The subjects in the two states responded similarly.

The differences in calcium and phosphorus excretion in the urine as a result of the two treatments were computed and tested for significance. The treatment effect on both calcium and phosphorus excretion was highly significant. Treatment 1, in spite of an increased calcium retention, resulted in a significantly greater urinary excretion of this element, whereas treatment 2 caused a significantly higher urinary excretion of phosphorus.

SUMMARY

Calcium and phosphorus metabolism were studied on 17 college women from two states. Five subjects, two in Ohio and three in Minnesota, were maintained for a period of 8 weeks on a basal diet which supplied approximately 300 mg of calcium and 800 mg of phosphorus daily. A second group of 6 subjects, three in Ohio and three in Minnesota, were maintained on the basal diet for 4 weeks. This was followed immediately by a 4-week period during which the basal diet was supplemented by 1,200 mg calcium, making the total calcium intake 1,500 mg daily. A third group of 6 subjects, following the 4-

week period on the basal diet, received supplements of calcium and phosphorus, making the total intake 1,500 mg of calcium and 1,400 mg of phosphorus.

One week was shown statistically to be an adequate period for adjustment to the calcium and phosphorus levels in the basal diet. Negative calcium balances were observed on the basal diet, whereas more positive than negative phosphorus balances were noted.

Increasing the calcium intake from 300 mg to 1,500 mg without altering the phosphorus intake resulted in a statistically significant increase in calcium retention. Urinary calcium excretion also increased significantly. Phosphorus retention, however, was unchanged, but there was a decrease in the urinary excretion of this element.

When supplements of both calcium and phosphorus were added to the basal diet, calcium retention was somewhat greater than on the unsupplemented basal diet but was very much less than when the basal diet was supplemented with calcium alone. Phosphorus retention, on the other hand, was not influenced by these changes in calcium and phosphorus intake.

Within the limits of the calcium and phosphorus intake levels employed in this investigation, the amount of phosphorus in the diet would appear to be a factor of considerable importance in determining calcium utilization in adult subjects.

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THE ESSENTIAL AMINO ACID (EXCEPT TRYPTOPHAN) CONTENT OF COLOSTRUM AND MILK OF THE SOW[1]

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With increasing interest in the development of synthetic rations for raising young pigs, it is necessary that the amino acid content of sows' milk be known if satisfactory rations are to be devised. However, at the time of writing no reports are to be found in the literature dealing with the composition of sows' colostrum and milk in respect to their content of amino acids. The amino acid levels in the milk protein in other species have been studied quite extensively. For example, the amino acid content of human and cows' milk has been compared by Plimmer and Lowndes ('37), Beach et al. ('41), Williamson ('44), and Block and Bolling ('46). Sarkar et al. ('49) studied the variation in the essential amino acid content of colostrum and milk of cows with stage of lactation, while Kuiken and Pearson ('49) carried out a comparative study of the amino acid content of both cows' and ewes' colostrum and milk.

Colostrum supplies special substances which enhance growth and enable the newborn animal to withstand infection. In some species immune bodies may be transmitted through the placenta directly from the blood stream of the mother to the

[1] The data reported in this paper were taken in part from a thesis presented by Stanley E. Beacom in partial fulfillment of the requirements for the degree of Master of Science. Acknowledgments are made to D. R. Clandinin, A. R. Robblee and Miss R. Renner for invaluable assistance with the assays. This work was supported by a grant from the Prairie Regional Committee of the National Research Council of Canada.

circulation of the fetus. In swine, which have an epitheliochorial placenta, appreciable placental transmission does not occur (Arey, '46), and colostrum is practically the sole source of antibodies for the newborn animal. Foster et al. ('51) found that the γ-globulin in suckling pigs' plasma increased from as low as 1.3 to about 20 mg per milliliter during the first 24 hours postpartum. Electrophoretic analyses also indicated that the sows' whey underwent marked changes in composition in this period.

In addition to a difference in protein distribution, sows' colostrum is known to have a higher protein content than milk (Braude et al., '47; Bowland et al., '49). In order to determine whether part of the beneficial effect of the colostrum for the young pig may be associated with high levels of one or more of the essential amino acids, it was decided to determine the level of 9 of these amino acids in sows' colostrum and milk. It was also deemed important to study the variation in level of the various amino acids throughout the lactation period. The amino acids studied were those known to be essential for the growth of the rat, although Shelton et al. ('50) have shown tryptophan, lysine, methionine, isoleucine and threonine to be indispensable for the weanling pig.

EXPERIMENTAL

Samples of colostrum and milk were obtained from two Yorkshire sows (404C and 275A) in their second and 6th lactation periods, respectively. The sows were both milked on the day of parturition (at three hours and 10 hours post-farrowing), then on the third day, the first week and at weekly intervals until the litters were weaned at 8 weeks of age. The sows were milked manually following the injection of 1 ml of an extract of the oxytocic principle of the posterior pituitary [2] containing 10 I.U. of the oxytocic principle, a method similar to that first used in swine by Braude et al. ('47). The samples which were taken were "quick frozen" and stored until all

[2] Pitocin.

milkings had been completed, at which time they were thawed and the determinations for nitrogen and amino acids carried out.

Nitrogen was determined on 2-ml (specific gravity averaged 1.0210 gm per milliliter) samples by the Kjeldahl-Gunning-Arnold method as described by the Association of Official Agricultural Chemists ('47), using mercuric oxide as catalyst. The factor 6.38 was arbitrarily used to convert nitrogen to crude protein in the case of both colostrum and milk.

Twenty milliliters of milk and 100 ml of 2N HCl were placed in 250-ml erlenmeyer flasks covered with 50-ml beakers and heated in an autoclave for 5 hours at a pressure of 15 lb. per square inch, which was found to give the most complete hydrolysis of various treatments studied. These hydrolysates were made up to 200 ml and filtered. They now had a concentration of 1 gm in 10 ml and were stored in the refrigerator for amino acid analysis.

The amino acid content of the samples was determined microbiologically, the media used and assay technique followed being similar to those employed by Riesen et al. ('47). *Leuconostoc mesenteroides* P-60 was used to determine lysine (Dunn et al., '44); *Lactobacillus arabinosus* 17-5 to determine leucine and valine (Schweigert et al., '45), isoleucine (Sheldon et al., '48) and phenylalanine (Riesen et al., '47); and *Streptococcus faecalis* R to determine methionine (Riesen et al., '46), arginine and histidine (Stokes et al., '45) and threonine (Greenhut et al., '46). L amino acid standards were used in the assays for lysine, leucine, arginine and histidine, while DL standards were used in the other assays.

RESULTS

Results of amino acid assays (averages for two or three replicate assays with duplicates in each assay) are summarized in tables 1 and 2. Table 1 presents the data in milligrams of amino acid per gram of milk. Because changes in the amount and composition of the colostral and milk protein are large, the figures in table 2 are given which show the

TABLE 1

Amino acid composition of sows' colostrum and milk

TIME SAMPLED	PROTEIN	METHIONINE	ARGININE	THREONINE	HISTIDINE	ISOLEUCINE	PHENYLALANINE	VALINE	LEUCINE	LYSINE
	%		*mg/gm of milk*							
	Sow 275A									
3 hours	21.22	2.50	11.85	11.63	3.89	7.07	8.52	13.70	19.12	13.38
3 days	6.55	0.92	4.00	2.76	1.42	2.85	2.45	3.54	5.68	4.81
1 week	5.10	0.71	3.13	1.85	1.11	2.27	1.84	2.69	4.60	3.82
2 weeks	4.56	0.63	2.75	1.65	1.10	2.03	1.57	2.48	3.97	3.55
3 weeks	5.06	0.71	3.03	1.85	1.25	2.13	1.79	2.74	4.46	3.95
4 weeks	5.41	0.79	3.31	1.86	1.23	2.41	2.17	2.77	4.43	3.94
5 weeks	5.27	0.68	3.08	1.73	1.20	2.26	1.81	2.59	4.33	3.77
6 weeks	6.13	0.80	3.73	2.07	1.38	2.56	2.15	3.17	4.87	4.95
7 weeks	6.57	0.77	3.44	2.11	1.39	2.44	2.13	3.10	4.70	4.56
8 weeks	7.14	0.95	4.24	2.49	1.44	2.97	2.54	3.68	5.92	5.08
	Sow 404C									
10 hours	9.13	1.15	5.84	4.41	2.23	4.05	3.74	5.57	8.33	6.13
3 days	5.82	0.80	3.42	2.45	1.09	2.49	2.03	3.04	4.69	3.86
1 week	4.89	0.71	2.78	1.74	1.04	2.21	1.67	2.46	4.01	3.41
2 weeks	4.70	0.63	2.71	1.79	1.11	2.12	1.70	2.37	3.88	3.99
3 weeks	4.73	0.64	2.64	1.72	1.16	2.10	1.58	2.24	3.68	3.36
4 weeks	5.23	0.73	2.89	2.00	1.21	2.24	1.79	2.58	4.10	4.05
5 weeks	6.37	0.84	3.34	2.22	1.24	2.60	2.08	3.08	4.86	4.49
6 weeks	6.62	0.84	3.49	2.39	1.25	2.63	2.18	3.20	5.02	4.50
7 weeks	6.48	0.94	3.76	2.43	1.28	2.78	2.32	3.29	5.08	4.90
8 weeks	6.59	0.92	3.60	2.30	1.50	2.73	2.30	3.29	5.00	4.82

TABLE 2

Amino acid composition of sows' colostrum and milk

TIME SAMPLED	PROTEIN	METHIONINE	ARGININE	THREONINE	HISTIDINE	ISOLEUCINE	PHENYLALANINE	VALINE	LEUCINE	LYSINE
	%		% OF TOTAL PROTEIN							
	Sow 275A									
3 hours	21.22	1.18	5.58	5.48	1.83	3.33	4.01	6.46	9.01	6.30
3 days	6.55	1.41	6.10	4.22	2.17	4.34	3.74	5.40	8.67	7.34
1 week	5.10	1.40	6.14	3.63	2.17	4.45	3.61	5.28	9.02	7.49
2 weeks	4.56	1.37	6.03	3.61	2.40	4.44	3.43	5.43	8.69	7.77
3 weeks	5.06	1.41	5.98	3.66	2.48	4.20	3.54	5.41	8.80	7.79
4 weeks	5.41	1.47	6.12	3.44	2.28	4.46	4.02	5.13	8.01	7.28
5 weeks	5.27	1.28	5.84	3.29	2.29	4.29	3.44	4.91	8.22	7.16
6 weeks	6.13	1.31	6.09	3.37	2.25	4.17	3.51	5.18	7.94	8.07
7 weeks	6.57	1.28	5.23	3.20	2.12	3.71	3.23	4.71	7.15	6.94
8 weeks	7.14	1.33	5.94	3.48	2.02	4.16	3.57	5.15	8.29	7.12
	Sow 404C									
10 hours	9.13	1.26	6.39	4.82	2.45	4.43	4.10	6.11	9.12	6.72
3 days	5.82	1.38	5.88	4.21	1.87	4.28	3.48	5.22	8.06	6.63
1 week	4.89	1.46	5.69	3.57	2.12	4.53	3.41	5.03	8.20	6.97
2 weeks	4.70	1.33	5.77	3.81	2.35	4.50	3.63	5.04	8.25	8.48
3 weeks	4.73	1.35	5.59	3.64	2.44	4.43	3.33	4.74	7.78	7.10
4 weeks	5.23	1.40	5.52	3.82	2.32	4.29	3.43	4.93	7.85	7.73
5 weeks	6.37	1.32	5.24	3.49	1.95	4.08	3.26	4.83	7.63	7.05
6 weeks	6.62	1.27	5.27	3.61	1.89	3.98	3.30	4.84	7.59	6.79
7 weeks	6.48	1.45	5.80	3.75	1.98	4.29	3.58	5.08	7.84	7.55
8 weeks	6.59	1.40	5.47	3.49	2.27	4.15	3.49	4.99	7.59	7.31

amino acid composition in terms of the percentage of the milk protein which they make up. This places the data on a sounder basis for comparative purposes since, with decline in protein as lactation progresses, there is a concomitant decline in amino acid levels.

Table 1 indicates that early colostrum was high in crude protein, containing over 21% at three hours post-farrowing for sow 275A. The protein level fell off rapidly until it reached a low of 4 to 5% around the second and third weeks. This level rose gradually to between 6 and 7% by the end of the 8-week lactation period. This trend in protein level of sows' colostrum and milk agrees with the results reported by Bowland et al. ('49).

When expressed as milligrams per gram of milk (table 1), all amino acids reached a low level between the second and third week, corresponding to the low protein level. When expressed as per cent of protein, the picture was changed considerably, as is indicated in table 2 and discussed in the following paragraph.

The protein of sows' colostrum was found to be higher in threonine, phenylalanine, valine and leucine and lower in methionine and lysine than was milk protein. Histidine and isoleucine showed little variation throughout the lactation period, although they were slightly lower in three-hour colostrum than in later colostrum and milk. Arginine levels were different for the two sows, one showing more arginine in the colostral protein than in the milk protein while the other showed the opposite trend.

DISCUSSION

A comparison of the amino acid composition of sows' milk and colostrum with that of the colostrum and milk of the cow and ewe revealed that there are many similarities in trends. The data on amino acid content of cows' milk by Kuiken and Pearson ('49) agreed in general with those presented by Sarkar et al. ('49), although in some cases differences between amino acid content of milk and colostrum varied in magni-

tude. Both these groups of workers found the protein of colostrum to contain more threonine, valine and arginine than milk protein. The present authors' data on sows' colostrum reveal it to be higher in threonine and valine and also in phenylalanine and leucine than milk. In the case of arginine, however, the picture differed for the two sows, as mentioned earlier. It was found that colostrum protein had less lysine and methionine and, in the case of very early colostrum, less isoleucine than the milk protein. Isoleucine, however, appeared to remain relatively constant throughout the remainder of the lactation period. Sarkar et al. ('49) found colostrum protein in cows' milk to be lower in phenylalanine and leucine in addition to isoleucine and methionine. They found lysine and histidine levels to remain fairly constant in both colostrum and milk proteins, whereas in the present study of sows' milk protein it was found that histidine was the only amino acid to remain relatively constant, although isoleucine was constant after very early colostrum. Lysine showed much less variation in level between colostrum and milk than was shown by the majority of the other amino acids.

A study of table 3 will facilitate comparisons between the amino acid composition of colostrum and milk proteins for the cow, ewe and sow. Main differences are as follows:

1. When figures for both sows are averaged, general "up and down" trends are in agreement with those reported for the cow and ewe, except in the case of phenylalanine and leucine, which made up a larger proportion of the colostral than of the milk proteins from the sows used in the study.

2. Another point of interest is that these 9 essential amino acids made up 44% of the colostral proteins and 41% of the milk proteins whereas, in the case of both the cow and the ewe, close to 50% of both colostral and milk proteins have been reported as being made up of these same essential amino acids. Some of these differences between species could be accounted for on the basis of a larger percentage of non-protein nitrogen in the milk, and particularly in the colostrum, of

TABLE 3

Comparison of colostrum and milk of the cow, ewe and sow

	COW [1]				EWE [1]				SOW			
	Colostrum		Milk		Colostrum		Milk		Colostrum		Milk [2]	
	mg/gm	% of protein	mg/gm	% of protein	mg/gm	% of protein	mg/gm	% of protein	mg/gm	% of protein	mg/gm	% of protein
Protein, %	15.00		3.40		18.26		4.71		15.18		5.68	
Methionine	2.84	1.89	0.90	2.65	3.47	1.90	1.22	2.59	1.83	1.22	0.77	1.36
Arginine	6.36	4.24	1.20	3.53	7.25	3.97	1.54	3.27	8.85	5.99	3.25	5.72
Threonine	10.44	6.96	1.48	4.35	12.58	6.89	2.08	4.42	8.02	5.15	2.01	3.54
Histidine	3.62	2.41	0.92	2.70	4.57	2.50	1.32	2.80	3.06	2.14	1.24	2.18
Isoleucine	7.07	4.71	1.99	5.85	8.78	4.81	2.64	5.61	5.56	3.88	2.41	4.24
Phenylalanine	6.74	4.49	1.62	4.76	8.18	4.48	2.24	4.76	6.13	4.06	1.98	3.49
Valine	11.60	7.73	2.42	7.12	14.81	8.11	3.55	7.53	9.64	6.29	2.86	5.04
Leucine	13.59	9.06	3.13	9.20	17.64	9.66	4.62	9.81	13.73	9.07	4.56	8.03
Lysine	12.02	8.01	2.90	8.53	14.55	7.97	4.06	8.62	9.76	6.51	4.20	7.39
Total		49.50		48.69		50.29		49.41		44.31		40.99

[1] Kuiken and Pearson ('49).

[2] Omitting three-day transitional milk.

sows. At the present time there are no data available to indicate the adequacy of the conversion factor of 6.38 used in this study to calculate protein from total nitrogen.

The data of Smith and Greene ('47) and Smith ('48), which show immune proteins to be higher in leucine, valine and threonine, help to explain why the protein of sows' colostrum should be higher in these three amino acids, assuming colostrum to be higher in content of immune proteins. Why it was also found to be higher in phenylalanine is not explainable on the basis of the data available, except that it may be accounted for by species differences.

Based on the limited data available, a comparison of the amino acid content of sows' milk protein to that of ewes' and cows' milk protein shows that the former contains less of 8 of the 9 amino acids studied than cows' milk protein has been reported to contain, the one exception being arginine, which was more plentiful in the protein of sows' milk than in that of cows' milk. In this connection, however, it should be remembered that sows' milk is considerably higher in protein (averaging over 60% higher) than cows' milk. When the two milks are compared on an equal volume basis, the fact that sows' milk is higher in protein tends to compensate for this difference in amino acid level. If this is done, as in table 3, it will be seen that sows' milk was superior to that of the cow in all amino acids studied except methionine. On this same basis sows' and ewes' milks were, generally speaking, similar, but methionine was considerably lower and arginine considerably higher in sows' milk.

SUMMARY

The essential amino acid composition (except tryptophan) of sows' colostrum and milk was determined and comparisons were made between these findings and the data on amino acid composition of cows' and ewes' milk reported in the literature.

On the basis of total protein, sows' milk contained less of all amino acids studied, except histidine, than did cows' and ewes' milk. However, when amino acid composition is ex-

pressed in terms of milligrams per gram of milk, the milk of the sow was superior to that of the cow except in the case of methionine, and very similar to that of the ewe.

Colostral protein was found to be higher in threonine, phenylalanine, valine and leucine, and lower in lysine and methionine than was milk protein. Histidine showed little variation throughout the lactation period. Isoleucine was found to be lower in the very first colostrum but quickly became constant in both later colostrum and milk samples. Arginine levels of the milk of the sows showed conflicting trends.

Because sows' colostrum is high in leucine, valine and threonine, and because these three amino acids are known to constitute a large proportion of immune proteins, it is reasonable to assume that colostrum is high in antibodies. This emphasizes the value of colostrum in the nutrition of the very young pig.

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PLASMA VITAMIN A AND CAROTENE DETERMINATIONS IN A GROUP OF NORMAL CHILDREN [1]

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TWO FIGURES

(Received for publication May 1, 1951)

As one phase in the long-range study of a group of normal children being conducted by the Child Research Council in Denver, blood vitamin determinations were started early in 1948. In this progress report, the results of the plasma vitamin A and carotene assays to date will be given, and some of the implications of these results will be discussed.

EXPERIMENTAL

The blood studies are being conducted on a group of 95 children (42 boys and 53 girls), ranging in age from two days to 16 years. These children come primarily from "upper middle class" economic groups. None is from a low income family.

Blood samples are taken when the children come in for routine pediatric examination; therefore, they are not fasting samples. Kimble ('39) reported that fasting and postprandial bloods did not differ in vitamin A or carotene content. The effect of vitamin A concentrate will be discussed later. Analyses are done at two days, at one, two, and three months of age, at three-month intervals thereafter until the subjects are three years of age, and at 6-month intervals

[1] This study was aided by a grant from the Nutrition Foundation.

from three years on. Formerly, all samples were taken by finger puncture. Since September, 1949, samples on about half the children have been taken by venipuncture, since more blood was required for other studies. No difference in vitamin levels between peripheral and venous blood has been observed in this laboratory.

The blood used for vitamin assays is that sample on which sedimentation rate and hematocrit values were determined. The stoppered, amber glass tubes in which the blood is allowed to sediment are kept away from direct sunlight. Plasma vitamin levels were found not to change during these procedures. The micro method of Bessey et al. ('46) is used for all vitamin A and carotene determinations.

Dietary histories of 55 of the younger children, most of whom are under 7 years old, are taken by a nutritionist during home interviews with the mothers.[2] The usual pattern of amount and frequency of each food eaten is established. In addition, four 24-hour intakes are obtained — one during the interview and three on subsequent days — recorded and sent in by the mother. The daily intakes are used as a cross check on the history, and intakes of specific dietary elements are calculated from standard food value tables. Nutrition histories are taken monthly during the first 6 months of life and thereafter at three-month intervals.

RESULTS

Vitamin A

Since the distribution of plasma vitamin A levels is skewed, percentiles present the most accurate picture of trends and deviations. The 25th, 50th, and 75th percentiles by age found in this group of children are given in table 1, along with minimum and maximum values. It will be observed that a peak

[2] The authors wish to thank Miss Virginia Beal for her cooperation in making the nutritional information on these children available to us for comparison with their plasma levels. The complete nutritional data will be published separately.

in the median, or 50th percentile, occurs at 9 months to one year of age, after which the values drop gradually to level off at about 6 years of age. The majority of extremely high values occur in this infancy period. Only three values above 70 μg % have been observed in the children older than three years and 6 months.

TABLE 1

Plasma vitamin A levels by age

AGE (years-months)	NO. OF CHILDREN	VITAMIN A IN μg %				
		Minimum	PERCENTILE[1]			Maximum
			25	50	75	
2 days	13	5	13	*23*	38	46
0-1	20	11	20	*29*	45	72
0-2	20	18	27	*36*	52	213
0-3	20	21	33	*42*	58	120
0-6	17	17	37	*47*	62	100
0-9	22	23	39	*56*	68	125
1-0	21	32	42	*52*	74	113
1-3 to 1-6[2]	18	19	38	*47*	64	143
1-9 to 2-0	19	17	36	*44*	58	113
2-3 to 3-0	15	27	32	*41*	53	102
3-3 to 4-9	17	14	31	*39*	50	62
5-0 to 6-0	23	18	28	*36*	46	96
6-6 to 16-0	56	14	28	*35*	42	110

[1] The 50th percentile is the median value for each age group. For example, at one month of age, half of the children had vitamin A levels equal to or below 29 μg %, one-fourth had levels equal to or below 20 μg %, and one-fourth had levels above 45 μg %.

[2] In the age groups from 1-3 up, the number of determinations on each child ranged from one to 8, depending upon the age span. In the 6-6 to 16-0 group there were 278 separate analyses on the 56 children.

In this sample of children, the peak near one year is due almost entirely to the girls in the group, since the boys tend to maintain a reasonably constant level. The differences between the means (girls' mean always higher) are as follows: at 6 months, 14 μg; at 9 months, 25 μg; at one year, 27 μg; at one year three months, 22 μg. At ages 9 months and one

year, these differences are statistically significant, $p = 0.01$ and < 0.001, respectively. After this age period there is no significant deviation between the sexes in plasma vitamin A.

There was no clear-cut seasonal variation in the vitamin A levels observed.

There was no significant correlation between plasma level and dietary sources of vitamin A alone. There was, however, some correlation ($r = +0.38$)[3] between daily intake of vitamin A concentrate and plasma level. This small degree of correlation is chiefly due to the fact that very few values over 50 μg % were found in the children whose diets were not supplemented with vitamin A concentrate; i.e., 5 of 70 analyses (7.1%) in children not receiving vitamin A concentrate compared to 92 of 254 analyses (36.3%) in children who received concentrate.

Since none of the infants so far studied has been free of vitamin A supplementation throughout the first year and a half, it is unknown whether or not the peak in levels near one year of age is independent of the concentrate given. Lewis, Bodansky and Haig ('41) found that the daily administration of 17,000 units of vitamin A raised the plasma level of vitamin A in infants up to 6 months of age, but not in those from 6 to 18 months of age. Their blood samples were taken 48 hours after the last supplement was given. It does not seem unreasonable to assume that the plasma level would be elevated in a sample of blood drawn at the peak of absorption of the day's vitamin supplement. One might speculate further that infants (girls in particular) have less ability to remove vitamin A from the circulation than have older children.

There was no apparent difference in plasma levels between the children receiving the vitamin A supplement in oil and those receiving it in a water dispersion.

[3] Correlation coefficient, $r_{xy} = \dfrac{\Sigma xy/n - (m_x\ m_y)}{\sigma_x\ \sigma_y}$

Carotene

The 25th, 50th, and 75th percentiles and the minimum and maximum values by age found for plasma carotene are given in table 2. The levels rise to a sharp peak at one year of age, after which they drop off fairly rapidly until three years.

TABLE 2

Plasma carotene levels by age

AGE (years-months)	NO. OF CHILDREN	CAROTENE IN μG %				
		Minimum	PERCENTILE [1]			Maximum
			25	50	75	
2 days	13	18	26	*34*	48	61
0-1	20	14	26	*37*	60	111
0-2	20	7	26	*42*	80	135
0-3	20	18	32	*45*	95	157
0-6	18	49	115	*174*	256	337
0-9	22	166	189	*268*	349	592
1-0	21	183	254	*328*	373	672
1-3	18	123	207	*281*	333	581
1-6	18	109	183	*253*	316	452
1-9	19	96	162	*233*	298	377
2-0 to 2-3 [2]	14	52	154	*201*	271	391
2-6 to 2-9	14	64	134	*171*	227	285
3-0 to 4-0	15	84	122	*155*	201	281
4-6 to 5-6	19	56	114	*150*	190	304
6-0 to 7-0	25	40	114	*149*	188	299
7-6 to 8-6	19	61	115	*147*	184	297
9-0 to 10-0	19	54	106	*130*	178	286
10-6 to 11-6	19	51	95	*117*	159	283
12-0 to 13-0	22	63	91	*114*	150	272
13-6 to 16-0	15	61	98	*126*	155	258

[1] The 50th percentile is the median value in micrograms per cent of each age group.

[2] In the age groups beyond 2-0 two or three determinations on each child may be included. For example, there were 55 assays on the 19 children in the 4-6 to 5-6 age period.

The values then remain almost constant until about 9 years, when a further drop occurs.

There is a definite relationship between plasma carotene and intake of vitamin A (carotene) from plant sources. A

prediction formula for plasma carotene based on dietary sources has been worked out as follows:

$$X_o = 0.46\ I_o + 0.06\ I_3 + 86$$

where X_o = expected plasma carotene in μg %,

I_o = average daily intake of vitamin A from plant sources in I.U. per kilogram of body weight during the past three months, and

I_3 = average daily intake of vitamin A from plant sources in I.U. per kilogram of body weight during the interval from three months to 6 months ago.

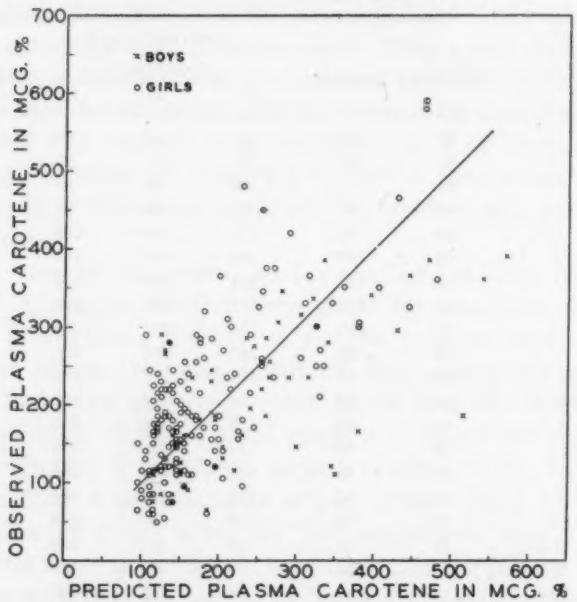

Fig. 1 Predicted plasma carotene level, based on dietary sources of carotene, is plotted against observed plasma carotene. The girls' values are represented by circles and the boys' by x's.

This formula was evolved from regression coefficients obtained from 40 observations of plasma carotene and dietary sources. The 40 cases were chosen on a basis of range of values and variation in intake from one three-month interval to the next. The correlation coefficient between actual and predicted carotene was + 0.85 on the original 40 cases and

+ 0.68 on the entire 228 sets of observations available. The latter are plotted in figure 1. It is expected that the relationship between intake and plasma level of carotene would be even closer if an adequate correction for absorption of carotene, depending upon the food source, could be made.

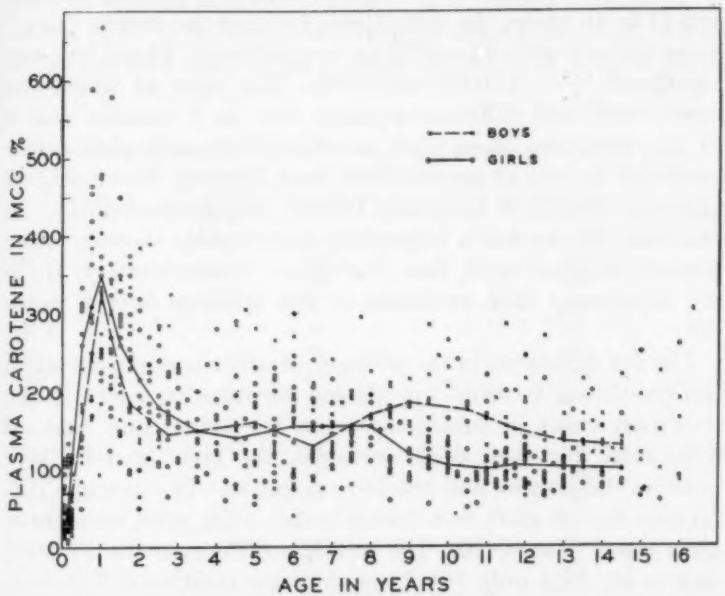

Fig. 2 The plasma carotene levels are plotted against age to show the sex difference in the infants and in the adolescents. Circles represent the girls' values and x's the boys' values. The solid line connects the girls' medians at different ages, while the broken line connects the boys' medians.

In two different age groups a marked sex difference in plasma carotene is present. The medians by age for boys and girls are graphed separately in figure 2. As in the case of vitamin A, the girls had higher carotene levels in infancy, despite slightly lower intakes of vitamin A from plant sources. After one year of age the groups were not significantly different with this number of children until about 8 or 9 years

of age. At this time the girls' values were depressed and the boys' showed a slight rise, followed by a drop at about 11 to 12 years. At ages one to three months, 6 months and 9 months the differences of the means (girls' mean higher) were 27, 101 and 91 μg, respectively. These differences are significant, $p = 0.008$, < 0.001 and 0.018. At ages 8 years to 10 years 6 months, and 11 to 16 years, the differences between the means (boys' mean higher) were 62 and 31 μg, respectively. These, too, are significant, $p = < 0.001$ and 0.032. The ages at which the most significant differences occur, one to 6 months and 8 to 11 years, are those ages at which boys and girls differ markedly in rate of growth (Dr. Jean Deming, University of Colorado School of Medicine, Denver, unpublished data). In each case the sex which is growing more rapidly shows a lower plasma carotene level than the other. Unfortunately, there are no dietary data available on the children over 8 years old.

The sex difference in the younger children is apparent when the prediction formula for plasma carotene is used. If the predicted value is subtracted from the actual one, and all differences for each child averaged, the girls tend to have positive differences and the boys negative. The average difference for 22 girls was found to be + 24, with only three girls lower than — 20. The average difference for 11 boys was — 46, with only two boys showing positive differences. Since this age group in the present experiments was heavily weighted with girls, the prediction formula applies more closely to the girls than to the boys. In figure 1 the majority of the boys fall below the theoretical line; that is, their observed plasma carotene levels are lower than would be predicted.

The seasonal variation in plasma carotene values is shown in table 3. In June through November the children over 4 years of age averaged 152 μg %, while in December through May these same children averaged 132 μg %. These averages cover three years of analyses. The difference is highly significant.

DISCUSSION

The vitamin A and carotene levels found in this group of children are similar to those reported by other investigators who used the Bessey and Lowry micro method for analyzing children's blood. Krause and Pierce ('47) found an average plasma vitamin A level of 38 μg % and plasma carotene level of 116 μg % in a series of Vermont school children. These levels are very close to the vitamin A median of 35 μg % found

TABLE 3

Seasonal variation in plasma carotene in children aged 4 to 16 years

MONTH	NUMBER OF DETERMINATIONS	PLASMA CAROTENE	
		μg %	
January	25	122	
February	34	136	
March	37	133	
April	33	140	
May	28	126	
June	21	152	
July	24	156	
August	30	147	
September	22	166	
October	43	158	
November	26	141	
December	19	123	
June–November	166	152	$p = < .001$
December–May	176	132	

in our group between the ages of 6 and 16 years, and to the carotene medians ranging from 114 to 130 μg % in the age groups older than 9 years. Robinson et al. ('48) reported average values ranging from 30 to 36 μg % vitamin A and 100 to 161 μg % carotene in children aged three to 18 years in various Michigan institutions. The same authors found an increase in the average vitamin A from 31 to 35 μg % and in the average carotene from 137 to 168 μg % in 6 weeks in a group of children attending a summer camp for underprivileged children.

Since vitamin A is stored by the liver and none of the children in this group was considered to be deficient in vitamin A, it is not surprising that there is little correlation between daily intake and plasma level of vitamin A. The most logical explanation for the high values found in infancy is probably the widespread use of vitamin concentrates. Yarbrough and Dann ('41) reported an average increase of 30% in the plasma vitamin A level in 16 adults two to three hours after a 20,000 I.U. test dose of vitamin A was given. Krause and Pierce ('47) found plasma vitamin A levels in children still elevated 24 hours after administration of 50,000 I.U. and 8 hours after giving 25,000 I.U. of vitamin A. Although the children studied here did not usually receive more than 12,500 units of vitamin A per day, it seems likely that this amount could account for the elevated plasma levels encountered frequently during the first two years of life.

The correlation between diet and plasma carotene would appear logical, since it is thought that carotene must first be converted to vitamin A before being stored by the body. It has been reported by Brenner and Roberts ('43) that the plasma carotene in 5 subjects on a low vitamin A diet dropped rapidly and leveled off after three months, while the plasma vitamin A remained constant. However, the applicability (in the present study) of a prediction formula, which contains constants representing the average daily intakes of vitamin A (carotene) from plant sources in both the preceding three-month and three- to 6-month periods, indicates the possibility of a more pronged effect of intake on blood carotene levels than is usually thought to be the case.

It is possible that the sex differences found in infancy in both plasma vitamin A and carotene and at puberty in plasma carotene are related to differences in rate of growth. Neither the exact nature of this relationship nor its dynamics are clear at present. In this connection it is of interest to note that Kimble ('39) found higher plasma carotene and lower vitamin A levels in women than in men. It is quite possible that

the boys and girls in this study will reach a similar relative position after adolescence.

In the absence of a vitamin A deficiency, the total significance of the plasma vitamin A level is not clear. In this group of 95 normal children there were wide variations between individuals as well as deviations in the same child from one examination to the next. The relationships between plasma vitamin A and age, sex, and intake of vitamin A concentrate indicate a few of the factors involved. The longitudinal study of individuals throughout the growth period is expected to provide many clues to possible correlations with physical, physiological, and biochemical changes. It will be possible, eventually, to compare the blood vitamin levels found in the individuals in this study with their nutrition, hematology, plasma proteins, basal metabolic rate, growth, functional tests, and general health.

Although plasma carotene is clearly related to dietary sources of carotene, and the evidence presented here suggests that it is related to growth rate, there are undoubtedly many factors influencing its level. In view of the sex differences observed, one might speculate that a slight shift in endocrine balance could influence the plasma carotene, or that an increased rate of growth is related to its more rapid conversion to vitamin A.

SUMMARY

The plasma vitamin A and carotene levels of 95 healthy children from two days to 16 years of age are described and the deviation with age shown.

Plasma vitamin A correlates slightly with the amount of vitamin A concentrate given, while plasma carotene shows a much higher correlation with intake of vitamin A from plant sources.

Infants show a significant sex difference in both vitamin A and carotene levels, the girls having higher values. Just before the start of adolescence in the girls the reverse sex

difference appears, but this time in plasma carotene only; that is, the girls have much lower values. As the boys approach adolescence later, their carotene levels also drop.

There is a significant seasonal variation in plasma carotene but not in vitamin A.

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WEST INDIAN AND NEW ORLEANS NEGRO METABOLISM TESTS

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The following study is based on 67 basal metabolism tests made upon West Indian Negro females with an average age of 39.79 years; 44 tests on New Orleans Negresses, average age 33; 63 tests on West Indian male Negroes, average age 32.45 years; and 50 tests on New Orleans male Negroes, average age 31 years.

The West Indian Negroes represent a fairly homogeneous group so far as height, body weight, physical types and appearance are concerned. The New Orleans Negroes were selected on the basis of presumable high degree of racial purity. Many were Delta Negroes, where mixture with other races has not been as prevalent as among the Negroes further inland. The West Indian Negroes show signs of inbreeding, if homogeneity and close similarity of external features are criteria.

The diet of the West Indians is largely salt cod, tea, bread, cocoa, and some beef. That of the New Orleans group includes fish, pork, coffee, and corn bread. Both groups frequently show signs of malnutrition evidenced by emaciation; some of their members appear to have suffered from rickets in childhood.

The West Indians are largely concentrated in towns; the groups under observation were residents of Charlotte Amalie on St. Thomas Island in the American Virgin Island group.

They exhibit many of the tensions observable in a highly industrialized community where living standards, because of economic pressure and competition for jobs, are low.

The records taken at New Orleans are largely from an agrarian population. While economic pressure and low living standards prevailed at the time of the study, the food habits were quite different from those prevailing in the Virgin Islands. Salt cod as part of the West Indian diet has been mentioned. This is commonly prepared, according to reports the author received from those using it, by tossing the cod caked with salt into a pot and cooking it without preliminary parboiling for removal of excess salt. A high incidence of hypertension was observed in those presenting themselves for the metabolism tests. Tests were not made on the hypertensives; some 6 individuals were excluded for this reason. Aside from these differences, a remarkable uniformity was observed in the various measurements taken from the New Orleans and the Virgin Island groups. The racial background of the West Indian and New Orleans Delta Negroes is probably similar. The West Indians appear to be in part the descendants of slaves left in the Virgin Islands during the time of the slave trade for reasons of sickness or because of physical unfitness, while the ancestors of the New Orleans Negroes, presumably recruited from the same tribes, were passed to the mainland of the United States.

In earlier studies by Crile and Quiring ('39a) on Chippewa Indians of the Churchill Bay Region and on Maya Quiché Indians of Totonicipan, Guatemala ('39b), the northern group, including 13 males and females, showed a deviation of $+ 18.25\% \pm 3.46$ from normal standards. The 20 Maya Quiché tests made on males indicated a deviation of $+ 8.20\% \pm 0.958$, based on normal standards.

Following is a tabular summarization (table 1) of the measurements and data gathered from the two groups represented in the present study.

TABLE 1

Comparisons of various measurements on West Indian and American Negroes

GROUP	BLOOD PRESSURE IN MM MERCURY		OXYGEN CONSUMED	HEIGHT	WEIGHT	AGE	PULSE PER MINUTE
	Systolic	Diastolic					
			ml/min.	*cm*	*kg*		
W.I. female	123.3	83.0	190.6	164.3	59.4	39.79	76.2
N.O. female	122.5	79.0	209.4	162.3	58.5	33.46	82.5
W.I. male	123.6	83.1	235.8	173.9	67.1	32.45	73.6
N.O. male	116.5	77.9	203.4	171.9	62.1	31.00	69.0

TABLE 2

Metabolism averages in percentages

GROUP	NUMBER AND + % MET.		NUMBER AND — % MET.		NUMBER 0%	NUMBER + 10% OR ABOVE	NUMBER — 10% OR BELOW
W.I. female	33	9.7	30	— 7.1	4	11	7
N.O. female	24	8.3	16	— 6.4	4	7	1
W.I. male	42	10.2	20	— 10.5	2	18	5
N.O. male	10	8.0	39	— 10.7	1	3	18
		ave.		ave.			
Total	109	+ 9.05	105	— 8.67	11	36	31

Since the tests on the two groups were made by the same observer using the same apparatus, the error factors due to individual peculiarities of technique should be similar.

The metabolism averages for the 4 groups are indicated in table 2.

While a number of values in each group fell either slightly above the + 10% or slightly below the — 10% limits, the averages for each group nevertheless remained within these boundaries.

Oxygen consumption was significantly higher in the West Indian male group and among the New Orleans females than it was in the New Orleans males and West Indian females.

TABLE 3

Calculated metabolism per square meter of surface area

GROUP	KgBW	ML PER MIN. PER KG	CALC'D. SA (M^2)	CALORIES PER 24 HR. PER M^2 SA
W.I. female	59.4	3.20	1.522	865.4
N.O. female	58.5	3.57	1.506	960.4
W.I. male	67.1	3.50	1.651	987.0
N.O. male	62.1	3.29	1.568	896.5

With this is associated a greater average body weight, amounting to 4.8 kg, in the West Indian males. The New Orleans male Negro uses 3.29 ml of oxygen per minute for each kilogram of body weight, while the West Indian male requires 3.50 ml per kilogram. The West Indian female requires 3.20 ml per kilogram and the New Orleans female Negro utilizes 3.57 ml.

The calculated surface areas, according to the Benedict ('32) formula, were closely similar for the entire group — ranging from one and one-half to almost one and two-thirds square meters. The calories produced per 24 hours, as will be observed in the following tabulations (table 3), were not far removed from 1,000 per square meter, and the findings roughly approximated the Bergmann-Rubner surface area

TABLE 4

West Indian females — means and deviations of various tests and measurements

NO. OF CASES	NATURE OF MEASUREMENT	MEAN	STANDARD ERROR OF MEAN	STANDARD DEVIATION	STANDARD ERROR	C.V.[2]	STANDARD ERROR C.V.
						%	
35	Diastolic B.P.	83.07 mm	± 1.60	9.45	± 1.14	11.38	± 1.36
35	Systolic B.P.	123.3 mm	± 3.38	20.0	± 2.38	16.22	± 1.93
67	O_2 consumed per minute	190.6 ml	± 2.75	22.55	± 1.86	11.28	± 0.99
35	Height distribution [1]	64.7 in. (164.4 cm)	± 0.429	2.54	± 0.303	3.92	± 0.468
35	Weight distribution [1]	131 lb. (59.4 kg)	± 1.80	10.64	± 1.27	8.12	± 0.971
35	Age distribution	39.97 yr.	± 0.537	3.17	± 0.38	9.07	± 1.08
35	Pulse per minute	76.23	± 1.58	9.35	± 1.11	12.25	± 1.46

[1] The calculations for these two sets of values were in inches and pounds.
[2] Coefficient of variation.

TABLE 5

West Indian males — means and deviations of various tests and measurements

NO. OF CASES	NATURE OF MEASUREMENT	MEAN	STANDARD ERROR OF MEAN	STANDARD DEVIATION	STANDARD ERROR	C.V.[2]	STANDARD ERROR C.V.
						%	
33	Diastolic B.P.	83.15 mm	± 1.777	10.20	± 1.255	12.28	± 1.509
33	Systolic B.P.	123.57 mm	± 2.56	14.70	± 1.89	11.81	± 1.45
65	O_2 consumed per minute	235.84 ml	± 3.68	29.50	± 2.58	12.08	± 1.05
33	Heights [1]	68.5 in. (173.9 cm)	± 0.465	2.67	± 0.329	3.91	± 0.49
33	Weights [1]	148 lb. (67.1 kg)	± 4.57	26.28	± 3.22	17.75	± 2.18
33	Age	32.45 yr.	± 2.02	11.85	± 1.45	36.82	± 4.53
37	Pulse per minute	73.61	± 1.406	8.55	± 0.991	11.61	± 1.346

[1] The calculations for these two sets of values were in inches and pounds.
[2] Coefficient of variation.

TABLE 6

New Orleans females — means and deviations of various tests and measurements

NO. OF CASES	NATURE OF MEASUREMENT	MEAN	STANDARD ERROR OF MEAN	STANDARD DEVIATION	STANDARD ERROR	C.V.[2]	STANDARD ERROR C.V.
						%	
22	Diastolic B.P.	79.0 mm	± 1.081	4.912	± 0.740	6.12	± 0.951
22	Systolic B.P.	122.5 mm	± 3.573	16.760	± 2.527	13.68	± 2.063
44	O_2 consumed per minute	209.4 ml	± 2.832	18.79	± 2.003	8.97	± 0.956
22	Heights [1]	63.9 in. (162.3 cm)	± 0.364	1.708	± 0.257	2.67	± 0.402
22	Weights [1]	129.2 lb. (58.5 kg)	± 3.302	15.495	± 2.336	11.21	± 1.690
22	Age	33.46 yr.	± 2.769	12.996	± 1.959	39.00	± 5.894
22	Pulse per minute	82.50	± 2.464	11.575	± 1.745	14.02	± 2.114

[1] The calculations for these two sets of values were in inches and pounds.
[2] Coefficient of variation.

TABLE 7

New Orleans males — means and deviations of various tests and measurements

NO. OF CASES	NATURE OF MEASUREMENT	MEAN	STANDARD ERROR OF MEAN	STANDARD DEVIATION	STANDARD ERROR	C.V.[2]	STANDARD ERROR C.V.
						%	
25	Diastolic B.P.	77.90 mm	± 1.36	6.80	± 0.943	8.72	± 1.245
25	Systolic B.P.	116.50 mm	± 4.00	20.00	± 2.85	17.16	± 2.45
50	O_2 consumed per minute	203.40 ml	± 2.44	17.13	± 1.713	8.42	± 0.842
25	Heights [1]	67.7 in. (171.9 cm)	± 0.58	2.92	± 0.431	4.31	± 0.630
25	Weights [1]	137.4 lb. (62.3 kg)	± 3.54	17.70	± 2.53	13.61	± 1.94
25	Age	31 yr.	± 2.11	10.55	± 1.507	34.00	± 1.65
25	Pulse per minute	69.00	± 1.60	8.00	± 1.14	11.59	± 1.65

[1] The calculations for these two sets of values were in inches and pounds.
[2] Coefficient of variation.

— metabolism law justifiably criticized by Benedict ('32), DuBois ('36) and others, but closely applicable in this instance.

The statistical tabulations for all the recorded data, together with calculated standard errors, are given in tables 4 through 7.

SUMMARY

Age, weight, height, temperature, blood pressure, pulse and metabolism data are offered for 67 West Indian female Negroes, 44 New Orleans Delta female Negroes, 64 West Indian male Negroes and 50 New Orleans Delta male Negroes.

Remarkable uniformity in blood pressure, height, and weight was exhibited in the two groups, although they live under quite different economic conditions. No significant differences in these characteristics were revealed.

The average metabolism rates fell within the limits established as normal for a white population.

The metabolism, in calories per square meter of calculated surface area, ranged from 865.4 cal. in the West Indian females to 987.0 cal. in the West Indian males; the New Orleans females had a metabolism value of 960.4 cal. and the New Orleans males 896.5 cal., per square meter of calculated surface area.

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THE RELATIVE EFFECT OF METHIONINE SULFOXIMINE ON DIFFERENT ANIMAL SPECIES [1]

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THREE FIGURES

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INTRODUCTION

During the past 15 years there has been considerable interest in the canine disease called running fits. Mellanby ('46) associated this condition with flour treated with agene (NCl_3), a compound which at one time was used to mature flour milled in the United States and England. Newell et al. ('47, '49a, b) have shown that there is considerable variation in the sensitivity of various animal species to agene-treated foods. Dogs, rabbits, ferrets, mink and cats develop fits when fed high levels of agenized materials. However, rats, chickens, guinea pigs, monkeys and humans do not show any sensitivity when fed similar rations. Recently three groups of workers (Bentley et al., '49; Reiner et al., '50a; Campbell et al., '51) have isolated the toxic factor in agenized flour. Two of these groups (Misani and Reiner, '50; Bentley et al., '50) have synthesized it, and report it to be a methionine derivative which Reiner has named methionine sulfoximine. Reiner has graciously made synthetic methionine sulfoximine prepared from DL-methionine available to us for further investigations.

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We wish to thank Dr. W. E. Gilson and Miss Hulda Gieschen for their assistance in recording and interpreting the electroencephalograms.

The experiments reported here are an attempt to study the effect of methionine sulfoximine on dogs, rats and monkeys on a more quantitative basis than has heretofore been possible.

EXPERIMENTAL AND RESULTS

Adult Sprague-Dawley rats which had been raised on stock laboratory rations were placed in individual cages and injected intraperitoneally with varying amounts of methionine sulfoximine. The results of this experiment are shown in table 1. Those rats which demonstrated the effects of methionine sulfoximine toxicity did so approximately 6 hours after being injected. The condition of these animals returned to normal 18 to 24 hours after the administration of the methionine sulfoximine.

TABLE 1

The effect of methionine sulfoximine injection in rats

NO. OF RATS	MG OF METHIONINE SULFOXIMINE/KG OF BODY WEIGHT	RESULTS
6	25	No abnormalities observed.
3	50	No abnormalities observed.
5	100	No abnormalities observed.
2	150	No abnormalities observed.
6	200	5 of the animals became hyperirritable; 3 of these became ataxic. 1 rat remained normal.
1	400	Animal became hyperirritable and ataxic. Convulsions were observed.

A number of healthy mongrel puppies in a post absorptive state were injected subcutaneously with varying amounts of methionine sulfoximine. Two dogs which received 1 mg of methionine sulfoximine per kilogram of body weight remained normal. Of 4 dogs which were injected with 3 mg of methionine sulfoximine per kilogram of body weight, three became convulsive within 48 hours and two of these died 5 days after the administration of the convulsant. No convul-

sions were observed in the 4th animal, but it refused food for 5 days. All dogs which were injected with 5 mg of methionine sulfoximine per kilogram of body weight became convulsive within 24 hours and all of them died two to 5 days after the injection.

Because of their phylogenic relationship to man, we were particularly interested in studying the effect of methionine

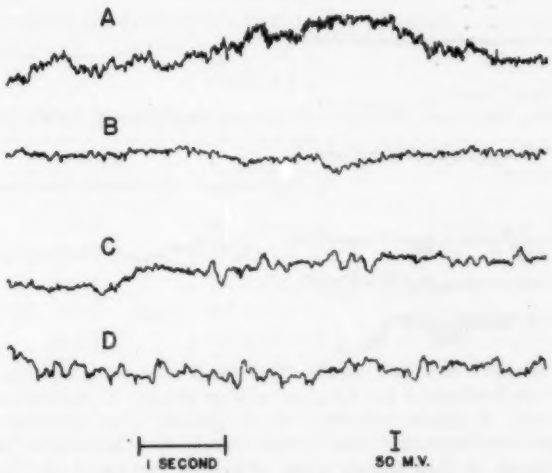

Fig. 1 Samples of E.E.G. records of monkey 3 after being injected with 250 mg of methionine sulfoximine per kilogram of body weight. A. Normal control; B. two hours after injection — normal; C. 5 hours after injection — some slowing of frequency, otherwise normal; D. 24 hours after injection — no change.

sulfoximine on monkeys. Intraperitoneal injections of 50 and 100 mg methionine sulfoximine per kilogram of body weight were without observable effect on monkeys in a post absorptive state. One monkey injected with 250 mg of methionine sulfoximine per kilogram of body weight stopped eating for 24 hours and its electroencephalographic record (fig. 1) showed some slight changes. Erickson et al. ('46) have reported the electroencephalogram as being "a most sensitive

and reliable indicator of the disturbance produced by feeding wheat gluten to dogs." Monkeys injected with 500 and 1000 mg of methionine sulfoximine per kilogram of body weight became ataxic and showed severe tonic and clonic convulsions within 4 hours. The seizures were followed by loss of consciousness. These animals showed abnormal electroencephalograms. Figure 2 represents the electroencephalogram pattern of a monkey which received 1 gm of methionine sulfoximine

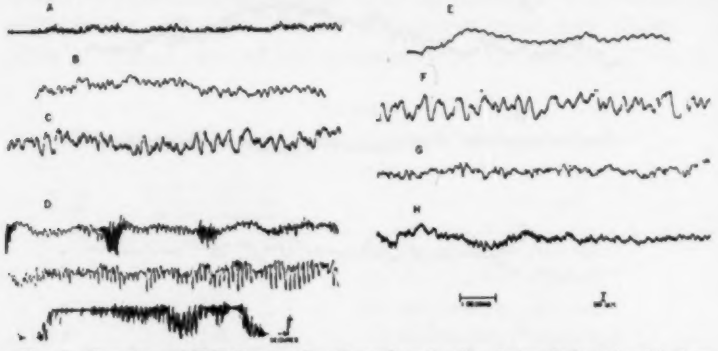

Fig. 2 Samples of E.E.G. records of monkey 2 after being injected with 1 gm of methionine sulfoximine per kilogram of body weight. A. Normal control with large amount of muscle potential; B. 30 minutes after injection — normal; C. 4 hours after injection — high voltage waves; D. 9 hours after injection — frequent bursts of high voltage spikes with basic rhythm of 10–12/second decreasing in frequency and increasing in amplitude until seizures occur; E. 9½ hours after injection — post ictal stupor activity; F. 23 hours after injection — similar to record C; G. two days after injection — return to normal; H. 8 days after injection — normal.

per kilogram of body weight. Seventy-two hours following the injection, the condition of this monkey returned to normal. Recently, Heathcote ('49) reported that the toxic factor in agenized proteins inhibited the growth of *Leuconostoc mesenteroides* P-60. Newell and Carman ('50) reversed this inhibition with methionine, and Reiner et al. ('50b) have suppressed the development of methionine sulfoximine-induced convulsions in rabbits and mice with methionine. We have completed similar experiments on dogs and monkeys.

Ten mongrel puppies in a post absorptive state were injected with 5 mg of methionine sulfoximine per kilogram of body weight. At different times before and after these injections varying amounts of methionine in capsule form were fed to these animals. The results of these experiments are shown in table 2.

It is apparent from this work that the administration of a sufficient amount of methionine to dogs shortly before or after they are injected with a toxic dose of methionine sulfoxi-

TABLE 2

The protective effect of methionine on dogs injected with methionine sulfoximine

NO. OF DOGS	METHIONINE / METHIONINE SULFOXIMINE	TIME OF METHIONINE ADMINISTRATION	RESULTS
2	50/1	1 hr. before inj.	Convulsions, death.
2	100/1	1 hr. before inj.	Complete protection.
1	150/1	1 hr. before inj.	Complete protection.
1	250/1	1 hr. before inj.	Complete protection.
2	100/1	1 hr. after inj.	Complete protection.
2	200/1	24 hr. after inj.[1]	Convulsions continued 1 dog died.

[1] These dogs were convulsive at the time of methionine administration.

mine will suppress the symptoms of running fits. However, once methionine sulfoximine-induced convulsions occur, the administration of methionine has little curative effect.

In another experiment a monkey was injected with 500 mg of methionine sulfoximine per kilogram of body weight. One and one-half hours before the injection this monkey was fed an amount of methionine equal to 9 times the quantity of methionine sulfoximine administered. This amount of methionine completely protected the animal from the effects of methionine sulfoximine toxicity. The electroencephalogram of the monkey receiving this treatment is presented in figure 3.

DISCUSSION

These experiments show that the reported variations in species sensitivity to agenized proteins are mainly quantitative in nature. The rat and monkey are approximately 100 times more resistant to methionine sulfoximine than the dog. The relatively rapid remission of the symptoms of toxicity in the monkey and rat indicate that they probably are able

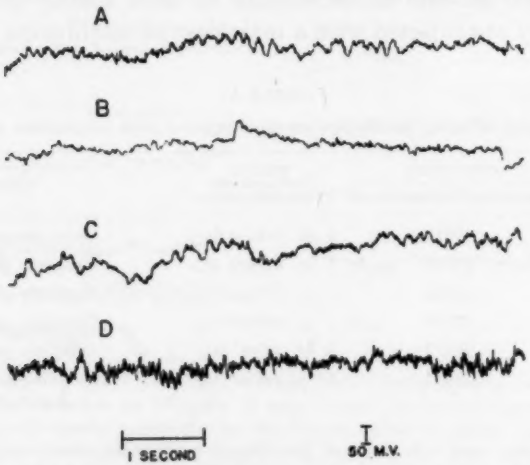

Fig. 3 Samples of E.E.G. records of monkey 4 after being fed 4.5 gm of methionine per kilogram of body weight and injected one and one-half hours later with 0.5 gm of methionine sulfoximine per kilogram of body weight. A. Normal control; B. two hours after injection — normal with movement artefacts; C. 5 hours after injection — normal; D. 24 hours after injection — normal with muscle potential.

to detoxify or excrete methionine sulfoximine more easily than the dog. This observation is now being investigated more thoroughly.

The treatment of flour with agene has been discontinued in the United States. However, the question of whether the use of commercially agenized flour has had a deleterious effect on the health of Americans remains unanswered. It has been clearly shown (Newell et al., '49a) that the feeding of

large amounts of highly agenized materials to humans, including some epileptics, for periods of up to 7 months will not result in the development of any abnormalities of dietary origin. There is a possibility that the continued use of commercially agenized flour over a period of years might lead to a cumulative toxicity, or that some people might be abnormally sensitive to it. However, no cases of this sort have been reported in the literature. The average American diet contains approximately 30% wheat products. When dogs, which are highly sensitive to methionine sulfoximine, were fed diets containing 30% commercially agenized flour for periods of up to 14½ months, neither running fits nor electroencephalographic abnormalities developed (Newell et al., '48). There is evidence that the resistance of man to methionine sulfoximine is equal to or greater than that of the monkey. Approximately 250 mg of methionine sulfoximine per kilogram of body weight are necessary to produce the slightest symptoms of toxicity in a monkey. When flour is exhaustively treated with agene, less than 5% of the methionine present is converted to methionine sulfoximine. If a 70-kilogram man were to be fed 500 gm (2,000 Cal.) of exhaustively agenized flour per day, his methionine sulfoximine intake would be less than 1 mg/kg of body weight/day. Furthermore, the methionine-methionine sulfoximine ratio in such a diet would probably preclude the possibility of toxicity.

SUMMARY

The minimum toxic dosages of methionine sulfoximine for dogs, rats, and monkeys have been established and it has been found that rats and monkeys are approximately 100 times more resistant to this drug than dogs.

The development of methionine sulfoximine-induced seizures in monkeys and dogs has been suppressed by the administration of methionine. The approximate ratio for the amount of methionine which will prevent seizures to the amount of methionine sulfoximine which will cause them has been

determined for dogs and monkeys. This ratio has been found to be considerably higher for the dog than for the monkey.

The relationship of these data to results already published dealing with the possible deleterious effect of commercially agenized flour on humans has been discussed.

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BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1952 Award of $1000.00 and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the Jury of Award may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1952. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 1, 1952. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

HARRY J. DEUEL, JR.
University of Southern California Medical School
Los Angeles, California

CHAIRMAN, NOMINATING COMMITTEE

MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1952 Award of $1000.00 established by Mead Johnson and Company to promote researches dealing with the B-complex vitamins. The recipient of this Award will be chosen by a Jury of Award of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1952.

The Award will be given to the laboratory or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Jury of Award, nominations for this award for work published in 1951 must be in the hands of the Chairman of the Nominating Committee by January 1, 1952. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Jury of Award in its consideration of the nomination.

SAMUEL LEPKOVSKY
University of California
Berkeley, California

CHAIRMAN, NOMINATING COMMITTEE

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1952 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 1, 1952.

HENRY A. MATTILL
Department of Biochemistry
Iowa State University
Iowa City, Iowa

CHAIRMAN, NOMINATING COMMITTEE